THE UNIVERSITY
of EDINBURGH

Nucleolar stress stimulates the NF-kappaB pathway: Mechanism underlying the pro-apoptotic effects of aspirin

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2017

Declaration

This is to certify that the work is entirely my own and not of any other person, unless explicitly acknowledged (including citation of published and unpublished sources). The work has not previously submitted in any form to the University of Edinburgh or to any other institution for assessment for any other purpose.

Signed 

Jingyu Chen

Data _____03/10/2017_____

To my family

Acknowledgements

I would like to take this opportunity to extend my sincere thanks to all the people who generously supported me during my PhD study.

First of all, I would like to express my deepest thanks to my PhD supervisor, Dr Lesley Stark, for all the helps and supports she gave me all through the years. Not like the 'typical' scientists, she is really close to students and always let me feel she is my friend. She was almost available for me for any questions I had every day. It was so helpful and efficient for my study that I can discuss with her any time when I had a problem or question in my study. Because of this, I felt I was improving every week and learned a lot during my study. She is super patient as well. It was difficult for me to adjust the life here when I first came to Edinburgh three years ago, as it was the first time I left my country and went abroad. She helped me settled in the new accommodation and invited me to her house to introduce me some Scottish culture. It was a great pleasure and superior experience to have an opportunity to work with Lesley during these years. It will be my life-long memory.

I am also grateful to my second supervisor Dr. Andy Finch (ECRC), and my PhD committee members, Professor Kathryn Ball (ECRC) and Dr. Juan-Carlos Acosta (ECRC). Thanks for their precious time in my committee meetings and anytime I had questions. I benefited a lot from their feedbacks and enlightening advice during my study.

I would also like to give my special thanks to Jim Simpson, Dr. Ian T. Lobb, Dr. Andrew O'hara and Dr. Pierre Morin. These former lab members had provided me enormous help and support during the time. Jim and Ian had done many fantastic works that form the fundament of my PhD thesis. Andy and Pierre helped me settle into the lab, and shown me all the routine lab protocols.

In addition, I want to express my sincere thanks to the College of Medicine and Veterinary Medicine that financially supported me through out my PhD study.

Finally, and importantly, I would like to thank my parents, Jiyin Chen and Qingxue Ding, who have being supported me without saving through out these years and encouraged me to be a better person. Thanks my wife, Yun Xu, and my lovely daughter Siqi Chen, with love and accompany of you all through this most exciting period of my life in Edinburgh, I was always full of passion, enthusiasm and confidence to work harder and achieve more. The endless loves and supports from my family means the whole world for me.

Abstract

The nucleolus is a multifunctional organelle that, in addition to its primary role in ribosome biogenesis, has emerged as a critical stress sensor and coordinator of stress response. However, the molecular nature of how nucleoli sense stress and coordinate downstream cellular consequence remains poorly understood. NF-κB signalling is a critical regulator of stress response. Many cellular stresses that disrupt nucleolar function also stimulate the NF-κB pathway. However, the role of NF-κB as a downstream effector of nucleolar stress has not yet been examined. Aspirin, a known chemopreventative agent, stimulates the NF-κB pathway to mediate apoptosis but the upstream mechanisms are unclear. In this thesis, I identified a novel nucleolar stress response pathway that culminates in activation of NF-κB signalling, and demonstrated the significance of this nucleolar pathway in the anti-tumour effects of aspirin. Using multiple approaches, I made the novel observations that disruption of the Pol I complex activates the cytoplasmic NF-κB signalling pathway. I show that multiple stress stimuli of NF-κB pathway induce degradation of the crucial Pol I complex component, rDNA transcription initiation factor IA (TIF-IA). I identified the tumour suppressor, p14ARF and the Pol I complex component, upstream binding factor (UBF) as mediators of this degradation. I revealed that inhibition of CDK4 activity lies upstream of UBF/p14ARF-facilitated TIF-IA degradation. Furthermore, using different approaches I show that blocking aspirin/CDK4i-mediated degradation of TIF-IA blocks the effects of these agents on nucleolar morphology and NF-κB signalling. Finally, I show this nucleolar stress response pathway, containing a UBF/p14ARF/TIF-IA axis, is utilized by aspirin to kill colon cancer cells. Taken together, this data presented in this thesis advances understanding of nucleolar stress response, and has therapeutic implications with regard to the anti-tumour effects of aspirin.

Key words: Nucleolus, TIF-IA, NF-κB pathway, p14ARF, UBF, aspirin, colorectal cancer

Lay Summary

The nucleolus, a cell compartment in the nucleus, is essential for cell growth. It is dysfunctional in the majority of cancers and as such, is emerging as a target for cancer therapeutics. Cells are surrounded by a rapidly changing environment and exposed to numerous stress signals. The nucleolus acts as a central hub for converting these environmental stress signals to alterations of cellular function, but the mechanism underlying this role of nucleolus is largely unknown. Accumulating data from clinical trials have provided optimism regarding the potential role of aspirin as a cancer preventative agent, especially against colon cancer. However, the potential harm from aspirin-induced bleeding and the unclear mechanism of the anti-tumour action of aspirin precludes its use as a chemopreventative agent.

This thesis aims to illustrate how the nucleolus coordinates cellular stress response, and explore the role of nucleolar regulation in the anti-tumour effects of aspirin, with a view to identify molecular targets for further development of anti-tumour drugs. Generally, I show aspirin and other cellular stress signals induce significant changes to the morphology of the nucleolus, changing its size and number. I also show aspirin inhibits nucleolar function by reducing the protein level of a critical nucleolar protein—TIF-IA, reduction of which has been associated with cell death. Using biochemistry methods, I have revealed the molecular basis of aspirin-induced TIF-IA degradation and demonstrated that modifying this pathway to block degradation of TIF-IA blocks aspirin's effects on nucleolus and colon cancer death. Therefore, in my PhD study, I define a cellular signalling pathway in the centre of nucleoli that is critical for the anti-tumour effects of aspirin in colorectal cancer. This could be an ideal target for developing novel anti-tumour agents that are safer and more effective.

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List of Abbreviations

AMPK	AMP-activated protein kinase
CK2	Casein kinase 2
CDK4i	Cyclin-dependent kinase 4 inhibitor
COX	Cyclooxygenase
CPT	Camptothecin
CRC	Colorectal cancer
DDR	DNA damage response
DFCs	Dense fibrillar centres
FCs	Fibrillar centre
GCs	Granular components
H_2O_2	Hydrogen peroxide
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
mRNA	message RNA
mTOR	Mechanistic target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NORs	Nucleolar organizer regions
NPM	Nucleophosmin

NSAIDs	Nonsteroidal anti-inflammatory drugs
P53	Tumor protein p53
Pol I	Polymerase I
PTMs	Post-translational modifications
rDNA	Ribosomal DNA
ROS	Reactive oxygen species
RPs	Ribosomal proteins
SILAC	Stable isotope labelling with amino acids in cell culture
SL1	The promoter selective factor
TIF-IA	Transcription initiation factor IA
UBF	Upstream binding factor

Chapter 1: Introduction

1.1 Nucleolus

The nucleolus is a distinct subnuclear organelle that is easily visible by phase contrast microscopy. In the past century, researchers have established the role of the nucleolus as the primary site of ribosome production and the mechanisms underlying the assembly, structure and function of nucleoli, and the pathways involved in ribosome biogenesis, have been comprehensively investigated.

1.1.1 Assembly, structure and function

The nucleolus was first formally documented between 1835-1839 by Wagner and Valentin independently. For the next century, due to the limitations of microscope technology, there were only occasional reports of the nucleolus in the broader context of cytological studies at that time. Then, from 1931-1934, Heitz and McClintock discovered that the nucleolus arises at a specific chromosomal locus. This discovery of nucleoli stationed at a genetic locus advanced nucleolar research in a way that proved nucleoli were cytogenetic entities rather than merely being an aspect of nuclear anatomy (Pederson, 2011). We now clearly know that nucleoli are not free-floating but anchored to specific genetic loci, which are termed nucleolar organizer regions (NORs), defined as clusters of head-to-tail tandem ribosomal DNA (rDNA) repeats. In humans, these repeat units are distributed on the short arms of five acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) (Figure 1.1). Only a proportion of NORs are transcriptional active in many cell types due to epigenetic modulation of proteins (such as histones) and chromatin at the rDNA loci (McStay and Grummt, 2008). A single NOR is capable to form a functional nucleolus, and nucleoli assembled on different NOR always display distinct size and shape. Besides, it often happens that several active NORs coalesce together to assemble one nucleolus (Savino et al., 2001). These properties of nucleolus assembly explain the diversity of this organelle.

Another criteria contributing to the diversity of nucleolar structure is that the nucleolus undergoes ordered assembly/disassembly depending on the stage of cell division. It is disassembled at the onset of mitosis and assembled again upon completion of mitosis (Hernandez-Verdun, 2011). Nucleolar assembly is dependent

upon transcription of rDNA by the Polymerase I (Pol I) transcription machine and therefore, only occurs at active NORs. The minimal molecular requirement to drive assembly of a functional nucleolus was firstly investigated by Karpen et.al in 1988. In a research, they found rDNA transcription and 'mininucleoli' formation could be driven when an ectopic rDNA sequence was introduced into the genome of Drosophila melanogaster (Karpen et al., 1988). This finding was further deciphered by Brain McStay's lab using a synthetic biology method. They artificially introduced DNA sequences from the ribosomal gene of Xenopus, which contained multiple binding sites for human upstream binding factor (UBF), into human chromosomes without a NOR. They found this rDNA fragment was sufficient to recruit endogenous UBF and other essential components of the Pol I transcriptional machinery. Importantly, these so-called 'pseudo-NORs' exhibited the same key features as an endogenous active NOR, although they were transcriptionally inactive and could not form functional nucleoli (Mais et al., 2005). They later expanded their finding by constructing a 'neo-NOR' which not only had a similar function to 'pseudo-NORs' but was also transcriptionally active, produced mature rRNAs and formed functional neo-nucleoli. This 'neo-NOR' cassette, which contained UBF-binding sites, human rDNA promoter and mouse rDNA transcription unit, can also coalesce with endogenous NORs to form larger nucleoli (Grob et al., 2014).

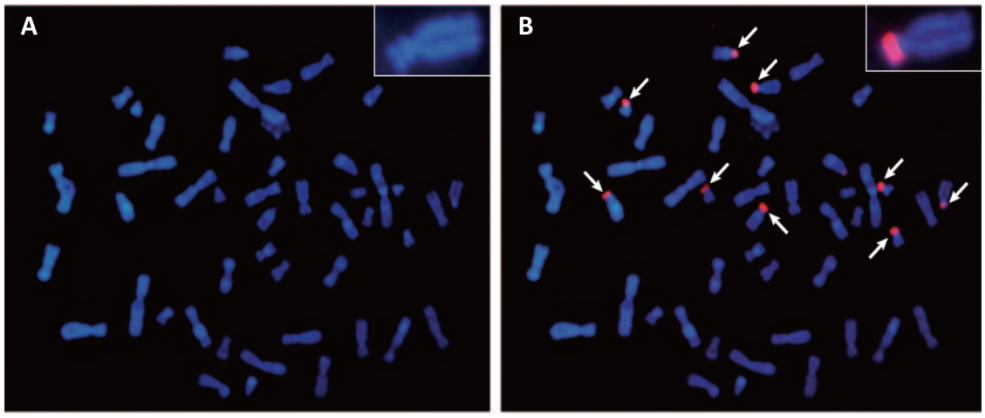


Figure 1.1 Nucleoli form around nucleolar organizer regions (NORs). NORs are clusters of head-to-tail tandem ribosomal DNA (rDNA) repeats, which are distributed on the short arms of five acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) in human. (A) shows the spread of all chromosomes with DNA stained by DAPI (blue) in metaphase human lymphocytes, right top inset highlight an acrocentric chromosome. (B) indicates NORs on acrocentric chromosomes by fluorescence *in situ* hybridization (FISH) with probe complementary to rDNA (red). Figure from Boisvert et.al, 2007

The notion that the nucleolus is the site of ribosomal RNA synthesis and ribosome assembly came to light in the 1960s. In a landmark nucleolus meeting at Montevideo, Uruguay in 1965, a report from Donald Brown and John Gurdon demonstrated nucleoli are responsible for ribosome biosysthesis as they found anucleoate Xenopus embryos exhibit impairment of ribosome production and arrested in development when the maternal stock of ribosomes was exceeded. This finding, along with other data presented in this conference demonstrating DNA complementary to ribosomal RNA (rRNA) residues in the nucleolus, and that rRNA is generated from larger precursor molecules, established the role of the nucleolus as a ribosome production factory (Pederson, 2011).

Following this meeting, the detailed molecular process of ribosome biosynthesis in the nucleolus was intensively addressed. In the past five decades, researchers have made significant progress in understanding the stages of pre-ribosomal particle assembly, determined a role for small nucleolar RNAs and ribonucleoproteins in rRNA processing and, with the help of electron microscopy, defined three distinct subregions of nucleoli. Basically, the nucleolus is tripartite in structure when observed under electron microscopy, which consists of fibrillar centres (FCs), dense fibrillar components (DFCs) and the surrounding granular component (GCs). As mentioned above ‘‘the nucleolus is an organelle formed by the act of building a ribosome’’, nucleoli locally concentrate the dedicated transcription and processing factors that are responsible for ribosome assembly, and the nucleolar tripartite structure is closely related to the complex and step-wise controlled ribosome assembly process. Each compartment reflects events related to ribosomal DNA transcription and ribosomal protein synthesis through enriching certain molecules involved in the process (Figure 1.2). Briefly, in FCs or at the boundary of FCs and DFCs, the initial 47S rRNA precursor is generated from Polymerase I (Pol I)-dependent transcription of rDNA clusters on the transcriptional active NORs. In the DFCs, 47S rRNA is cleaved to form 28S, 18S and 5.8S rRNAs, which then undergo steps of post-transcriptional modifications through interacting with a number of Polymerase II (Pol II) transcribed small ribonucleoproteins (snoRNPs). Finally, in the GCs, modified rRNAs are assembled with Pol III transcribed 5S rRNA and a multiple ribosomal proteins and assembly factors generate the large pre-60S

ribosome (28S, 5.8S, 5S rRNAs) and small pre-40S ribosome (18S rRNA) subunits. These then interact with the export machinery and are transported to the cytoplasm where the mature functional ribosomes are ultimately formed after binding with message RNAs (mRNAs).

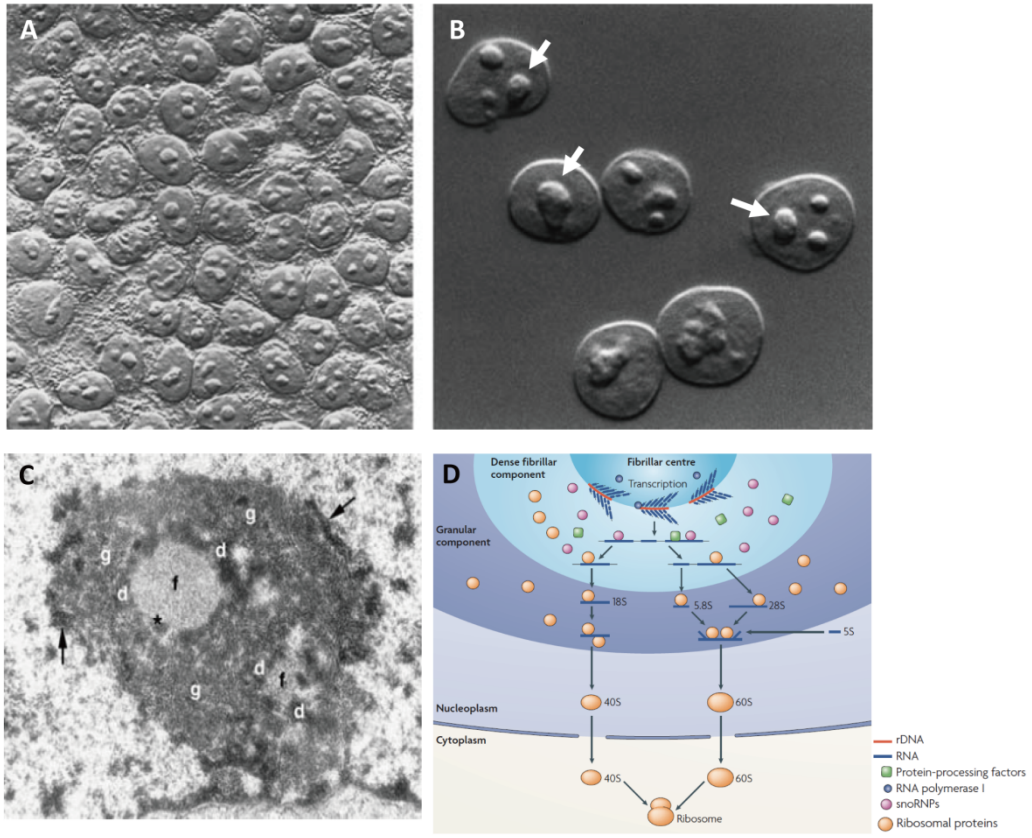


Figure 1.2 Visualisation, structure and function of the nucleolus. Differential interference microscopy (DIC) image of nucleoli in Hela cells (A) and nucleoli (arrows) in isolated Hela cell nuclei (B). (C) shows the tripartite organization of the nucleolus by electron microscope. f. fibrillar center; d, dense fibrillar component; g, granular component. (D) Model of the step-wise ribosomal biogenesis, which is spatially compartmentalised in the nucleolus. (A-C) from Thoru Pederson, 2011. (D) from Boisvert et.al, 2007.

1.1.2 rDNA transcription—the Pol I transcription machinery

The initiation of ribosomal biosynthesis requires the active transcription of ribosomal DNA, which is dependent on the assembly of Pol I transcription initiation machinery (a multi-protein complex incorporating RNA polymerase I) on the promoter region of rDNA genes.

1.1.2.1 The formation of Pol I transcription machinery

In mammals, binding of the two ancillary factors, upstream binding factor (UBF) and the promoter selectivity factor (termed SL1 in human, TIF-IB in mice), to the rDNA promoter, initiates the formation of the pre-initiation complex. UBF binds as a dimer to the upstream control element (UCE) and the GC-rich element in the core promoter of rDNA through its high mobility group (HMG) boxes. Like other sequence-nonspecific high mobility group proteins (such as HMG1 and HMG2), the presence of the HMG box allows UBF to bend DNA and act as an architectural element to maintain chromatin/chromosomes structure. The UBF dimer interaction with the UCE enables a single turn of ~140 base pairs of enhancer DNA to form a nucleosome-like structure called the enhancosome. The formation of the enhancosome juxtaposes the precisely spaced UCE and core promoter to structurally support the assembly of the pre-initiation complex. Upon DNA binding of UBF, the promoter selectivity factor SL1/TIF-IB can interact with UBF and bind DNA cooperatively. SL1/TIF-IB is a protein complex of the TATA binding protein (TBP) and TBP-associated factors including TAFI110/95, TAFI68, TAFI48, TAFI35 and TAFI12 (Drygin et al., 2010). It confers promoter specificity and provides a crucial link between the rDNA promoter and core polymerase Pol I, which itself has no DNA binding activity, by interacting with a number of Pol I associated factors (like TIF-IA/RRN3 and PAF53) to recruit Pol I to the promoter. Pol I itself is a complex component, which in human has two distinct isoforms Pol I α and Pol I β. Although Pol I α comprises ~90% of cellular Pol I, it is Pol I β involved in the transcription initiation complex while Pol I α may play a more important role in transcription elongation (Russell and Zomerdijk, 2005).

The assembly of the Pol I transcription pre-initiation machinery could be a stepwise process (subunits recruited to the promoter in order) or a pre-assembly process (all the factors assembled into a holoenzyme before docking at the promoter). It is still debatable with evidence for both hypotheses. The isolation and identification of a Pol I holoenzyme from mammalian (Hannan et al., 1999) (Seither et al., 1998) and Saccharomyces cerevisiae (Schneider and Nomura, 2004), containing Pol I and other core elements, has supported the scenario of a pre-assembly process. In contrast,

other reports have suggested the subunits are recruited to the promoter individually (Russell and Zomerdijk, 2005). In particular, experiments in live cells using fluorescence recovery after photobleaching (FRAP) methods have shown the individual dynamics of Pol I subunits and UBF as well as PAF53 and TIF-IA have distinct properties under conditions of different transcription levels (Gorski et al., 2008).

1.1.2.2 Regulation of Pol I transcription

The production of ribosomes is the most energy consuming process in a cell. Therefore, there is a tight link between ribosome biogenesis, protein synthesis, cell growth and cell proliferation. The efficiency of protein synthesis is largely limited at the level of rDNA transcription or by the content of cellular rRNA, which means cells need to maintain a high rate of rRNA production to supply enough ribosomes for cell growth and metabolism. Therefore, the activity of rDNA transcription is efficiently controlled under changing cellular environment. Considering the nature of ribosomal DNA (hundreds copies of tandemly repeated rDNA genes), there are two possible models proposed for transcriptional regulation of rDNA.

For long-term regulation, the number of transcriptionally active NORs engaged in active transcription is controlled at an epigenetic level through histone modification and DNA methylation (McStay and Grummt, 2008). In this process, several factors have been found to be involved in chromatin remodelling to alter the ratio of active to silent rDNA gene copies, including TTF-I (transcription termination factor I), CSB (cockayne syndrome protein B) and NoRC (nucleolar remodelling complex). It should be noted that UBF, as a member of Pol I transcription initiation complex, also plays an important role in regulating the condensation of chromatin structure through its HMG box DNA binding motifs (McStay and Grummt, 2008) (Grob et al., 2014; Mais et al., 2005).

The epigenetic regulation of rDNA transcription accounts for the majority of species-specific or tissue-specific transcription of rDNA genes, and functions throughout cell cycle progression, cell development and differentiation. However, given the high-energy demand of cell metabolism and dramatic changes in the environment a cell

can potentially face, a more rapid, short-term rDNA transcription regulation model is required for finely adjusting transcription rates. In this short-term regulation system, the transcription rate of an individual active gene is changed by reversible, post-translational modifications of Pol I transcription factors in response to various upstream signalling pathways. These modifications in turn affect the Pol I transcription initiation, elongation and rRNA processing and then results in different ribosomal production efficiency (Figure 1.3). For example, the PI3K/AKT and RAS/RAF/MAPK/ERK pathways are well-known to regulate rDNA transcription in response to growth factors while an mTORC1 regulated pathway is known to play a central role in monitoring nutrient availability and regulating the rate of rDNA transcription. AMP dependent protein kinase (AMPK) is the critical energy sensor that transmits the cellular energy status to rDNA transcription outcome. These pathways can target multiple modification sites on crucial Pol I factors, like UBF, SL1 and TIF-IA, to change the activity of Pol I transcription (Reviewed by (Kusnadi et al., 2015)).

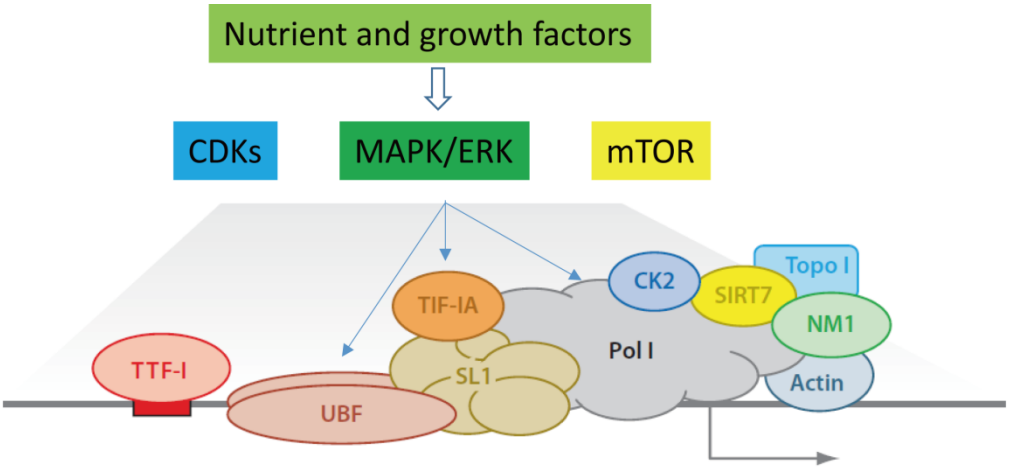


Figure 1.3 Basal components in the Pol I transcription machinery. Figure shows the basal factors associate with the promoter region of rDNA that are responsible for the initiation of rDNA transcription. Upstream pathways modulate crucial Pol I factors in response to nutrient or growth conditions are briefly indicated. Figure adapted from Drygin et.al, 2010.

1.1.2.3 Regulation of critical Pol I transcription factors

UBF

Synthetic biology assays have revealed UBF is the minimal component to drive formation of the basal Pol I transcription machinery and assembly of a functional nucleolus. Therefore, it is anticipated that any modulation of UBF activity would be coupled with dramatic effects on the Pol I transcription activity. The activity of UBF is controlled by regulated phosphorylation. It is phosphorylated at serine residue 484 by cdk4/cyclinD1 and cdk2/cyclin E upon serum stimulation (Voit et al., 1999) and phosphorylated at serine residue 388 by cdk2/cyclin E and cdk2/cyclin A during S phase of the cell cycle (Voit and Grummt, 2001). Under mitogenic stimulation, UBF is phosphorylated by ERK at threonine 117 and threonine 201 which are within HMG boxes 1 and 2 (Stefanovsky et al., 2001). The mTOR pathway is required for serum-induced activation of rDNA transcription via ribosomal protein S6 kinase 1 (S6K1) phosphorylation of UBF on the carboxy-terminal activation domain (Hannan et al., 2003). These reports suggest that phosphorylation of UBF results in variation of Pol I activity through affecting its interaction with other Pol I factors or rDNA. Reports also suggest acetylation of UBF affects its activity. Acetyltransferase CBP activates Pol I transcription by acetylating UBF and reversing its inhibition by tumour suppressor pRb and associated histone deacetylases (Pelletier et al., 2000).

SL-1

SL-1 is essential to initiate the formation of the pre-initiation complex. It is known that 'basal' levels of rDNA transcription in vitro can be supported by the presence of only SL1 and Pol I (Russell and Zomerdijk, 2005). During mitosis, Cdk1/cyclin B phosphorylates TAF_I110, a subunit of SL1/TIF-IB, on threonine 852, thus impairing the interaction of SL1/TIF-IB with UBF and silencing Pol I transcription (Heix et al., 1998). In a reconstituted *in vitro* system, p300/CBP-associated factor PCAF, a histone acetyltransferase, acetylates SL1/TIF-IB subunit TAF_I68 and promotes its DNA binding activity. In contrast, the histone deacetylase, mSir2a, deacetylates TAF_I68 and represses rDNA transcription (Muth et al., 2001). These suggest, in response to external signals, revisable acetylation on SL1 could be another effective way to regulate rDNA transcription activity.

TIF-IA

Transcription initiation factor TIF-IA, the mammalian homolog of yeast Rrn3, is a crucial member of the RNA polymerase I transcription machinery. Of all Pol I complex factors, TIF-IA is the critical component for responding to environmental cues and cellular stress. TIF-IA interacts with Pol I β, coordinating the interaction of Pol I and SL-1/TIF-IB, which is necessary for recruiting Pol I to the rDNA promoter and to assemble the transcription initiation complex (Yuan et al., 2002).

Since TIF-IA plays a crucial role in regulating growth-dependent rDNA transcription, disruption of TIF-IA always induces impairment of rRNA synthesis and generally leads to abnormal cell growth and apoptosis. Yuan X et.al inactivated the TIF-IA gene by Cre-mediated homologous recombination in mice. TIF-IA-/- embryos exhibited retardation of growth and development and died before or at embryonic day 9.5. They also discovered using induced deletion of TIF-IA in MEFs, that disrupting nucleoli, caused cell cycle arrest and high rates of apoptosis, as well as activation and stabilization of p53. These TIF-IA depletion effects were abrogated by knocking down p53 (Yuan et al., 2005). In another set of data where TIF-IA was specifically knocked out in the nervous system, TIF-IA-deficient mice displayed upregulated p53 levels in neural progenitors and hippocampal neurons and demonstrated neurodegeneration and dies shortly after birth (Kiryk et al., 2013; Kreiner et al., 2013; Parlato et al., 2008). These findings indicate that it is possible to induce cell suicide or tissue damage by ablation of TIF-IA.

TIF-IA is targeted by multiple protein kinases in response to environmental signals, which regulates the formation of the transcription initiation complex and accordingly, modulates cell growth and proliferation (Table 1.1). Following oxidative or ribotoxic stress, JNK2 was shown to phosphorylate TIF-IA at Thr200. This effect causes TIF-IA nucleolus to nucleoplasm translocation and results in inhibition of rRNA synthesis (Mayer et al., 2005). mTOR regulates two phosphorylation sites on TIF-IA, serine 44 and serine 199, under certain nutrient related stresses. Interestingly, mTOR mediated phosphorylation at these two sites has opposite effects. Inhibition of mTOR activity causes TIF-IA to translocate to the cytoplasm, impairs formation of the rDNA transcription initiation complex and suppresses rRNA synthesis (Mayer et al., 2004). AMP-activated protein kinase (AMPK) regulates TIF-IA under energy

depletion stress. In this case, AMPK phosphorylates serine 635 of TIF-IA leading to impairment of the interaction between TIF-IA and SL1 and down-regulation of Pol I transcription activity (Hoppe et al., 2009).

In addition to stress-related regulation, TIF-IA activity is regulated by growth factors (Table 1.1). These factors increase rRNA synthesis through activating mitogen-activated protein kinase (MAPK) phosphorylation of TIF-IA. Serine 633 and 649 on TIF-IA are targeted by ERK and RSK to transfer growth signals to rDNA transcription initiation and ribosomal subunit biogenesis (Zhao et al., 2003). Phosphorylation of serine 170/172 at TIF-IA by Casein Kinase 2 (CK2) is required for efficient rDNA transcription cycling. This phosphorylation triggers the switch from rDNA transcription initiation to elongation phase by disassociating TIF-IA from Pol I. FCP1 dephosphorylates these two sites and re-tethers TIF-IA to the Pol I polymerase to switch a new round rDNA transcription initiation (Bierhoff et al., 2008).

Besides phosphorylation, a few researches have shown basal TIF-IA is regulated by ubiquitination dependent degradation. Fatyol and Grummt demonstrated that TIF-IA associates with proteasomal ATPases and demonstrated that this may contribute to TIF-IA's functional ubiqutination-degradation (Fatyol and Grummt, 2008). Meanwhile, Le Xuan Truong Nguyen and Beverly found that the E3 ligase MDM2 co-localizes with TIF-IA in the nucleus and increases its ubiquitination and degradation (Nguyen le and Mitchell, 2013). However, the regulation of TIF-IA protein levels following cellular stress has never been reported.

Given that TIF-IA coordinates multiple extracellular signals to link rRNA synthesis with stress response and cell growth, it represents a promising molecular target for drugs designed to block cell proliferation (Drygin et al., 2010).

Sites	Upstream pathway	Effects	Reference
S44	mTOR	Activate TIF-IA	Mayer C, Genes Dev. 2004 Feb 15;18(4):423-34.
S199	mTOR	Inactivate TIF-IA	Mayer C, Genes Dev. 2004 Feb 15;18(4):423-34.
S170/172	CK2(phos.)	TIF-IA disassociation with PolI. rDNA transcription; Nucleolar integrity; Cell cycle progression.	Holger Bierhoff, et.al, Mol. Cell. Biol. 2008, 28(16):4988.
	FCP1(dephos)	TIF-IA associate with PolI again.	Holger Bierhoff, et.al, Mol. Cell. Biol. 2008, 28(16):4988.
	Akt (through CK2)	Preventing TIF IA's MDM2 -mediated ubiquitination and degradation; Enhances ribosomal RNA synthesis	Le Xuan Truong Nguyen, PNAS, December 17, 2013, vol. 110, no. 51, 20681–20686
T200	JNK	Inactivation of TIF-IA; Down-regulate rRNA synthesis; TIF-IA from nucleolus to nucleoplasm	Mayer C, Genes Dev. 2005 Apr 15;19(8):933-41. Epub 2005 Apr 1.
s635	AMP-activated protein kinase (AMPK)	Down-regulate rRNA synthesis	Hoppe et.al, PNAS. 2009 Oct 20; 106(42): 17781–17786.
S633, s649	MAPK, ERK, RSK	Activation of TIF-IA and ribosomal gene transcription	Zhao J, Mol Cell. 2003 Feb;11(2):405-13.

Table 1.1 Published phosphorylation modification sites on TIF-IA. Table summarises the known phosphorylation sites on TIF-IA, and upstream pathways targeting these sites that alter the activity of TIF-IA and ribosomal biosynthesis.

1.2 The plurifunctional nucleolus

A bold but prescient statement came out at the same time as the ribosome biosynthesis function of nucleolus unravelled. In a summary report of the historic Montevideo conference on the nucleolus in 1965, C.H.Waddington, an embryologist from Edinburgh, said: ‘‘The nucleolus probably should not be considered a relatively simple organelle with a single function, comparable to a machine tool turning out a particular part of an automobile. It is not just ‘the organelle where the cell manufactures ribosomes’. It is rather a structure through which materials of several different kinds are flowing, comparable more to a whole production line than to a single machine tool.’’(Pederson, 2011)

It was shortly after this statement that experimental data suggested the nucleolus has functions in addition to ribosome biogenesis. In the late 1960s and early 1970s, the Harris lab deduced that nucleoli participate in the biogenesis of certain messenger RNA. By fusing chicken erythrocytes containing a condensed inactive nucleus with transcriptionally active HeLa cells, they found that, despite the presence of HeLa cell

ribosomes in the cytoplasm, the new synthesized chicken specific proteins only became detectable in the heterokaryons after the inactive erythrocyte nucleus had undergone chromatin decondensation and nucleoli had been formed (Pederson, 2011).

Despite Harris's effort to promulgate the additional functions of nucleolus beyond assembly of ribosomes, the diversity of nucleolar functions was only really realised by the world in the early 1990s, when Thoru Pederson's lab discovered its role in the biosynthesis of the signal recognition particle (SRP). The SRP is a ribonucleoprotein complex that can bind to the signal sequence of nascent polypeptides to halt translation elongation. Subsequently, it is involved in the process of transporting new synthesized peptides to recognition components on the endoplasmic reticulum (ER) for secretory or membrane translocation (Jacobson and Pederson, 1998). The mature SRP is abundant in the cytoplasm. Therefore, Thoru Pederson lab supposed SRP RNA would not localize in nucleoli and would be a suitable negative control for a study to define nucleolar targeting RNAs. To their surprise, following the microinjection into the nucleus, the fluorescently labelled SRP RNA demonstrated a rapid but transient translocation into the nucleolus, before moving out of nucleolus to its conventional cytoplasmic location (Jacobson and Pederson, 1998). This finding, together with a number of other discoveries showing certain mRNAs were generated in the nucleolus rather than the cytoplasm, led Thoru Pederson to propose the 'plurifunctional nucleolus' hypothesis (Pederson, 1998). Further studies built on this discovery by demonstrating the distribution of SRP RNA within the nucleolus was distinct from the known stations of ribosome biogenesis, and revealing the nucleolus is indeed a site of SRP assembly (Sommerville et al., 2005). These discoveries therefore suggested there might be subnucleolar regions specialized for other function beside ribosome biogenesis.

The idea of the 'plurifunctional nucleolus' was significantly enhanced by work from the Lamond lab defining the nucleolar proteome. They first combined nucleoli purification and mass spectrometry (MS) to identify a group of nucleolar proteins, including some proteins without previously known nucleolar functions, and some proteins were encoded by uncharacterized genes at that time (Andersen et al., 2002). This proteomic data revealed more complicated nucleolar functionality than

previously thought. In addition, using the well-established technique of large-scale biochemical isolation of nucleoli combined with stable isotope labelling with amino acids in cell culture (SILAC)-based quantitative proteomics, they were able to demonstrate that human nucleoli contain over 4500 proteins, and the majority of which (70%) have functions outside of ribosome biogenesis (Ahmad et al., 2009). This further indicated a multifaceted role for the nucleolus as central hub to link ribosomal subunit biosynthesis with other cellular functions. Since these initial experiments, a wealth of information has been deduced regarding the nucleolar proteome from a number of different species and in response to different stimuli (Boisvert et al., 2012; Boisvert et al., 2010; Moore et al., 2011). Interestingly, a study from the lab comparing the human and yeast nucleolar proteomes found that ~90% of the nucleolus-associated yeast proteins have a human homologue (Andersen et al., 2005) indicating high conservation of the nucleolar proteome through evolution.

From proteomic studies, there are also some interesting findings been revealed, like the existence of a large pool of unassembled ribosomal proteins and ubiquitination and proteasome degradation of ribosomal proteins, which are beyond the conventional notion of ribosome biosynthesis in the nucleolus and reveal more surprises regarding nucleolar function (Pederson, 2011).

Moreover, proteomic data have implied the nucleolus is involved in the regulation of cellular functions that determine the cell fate (Figure 1.4). This have also been evidenced by a growing body of experimental studies, reinforcing the multifunctional role of the nucleolus in the regulation of cell cycle, cell apoptosis and cell senescence.

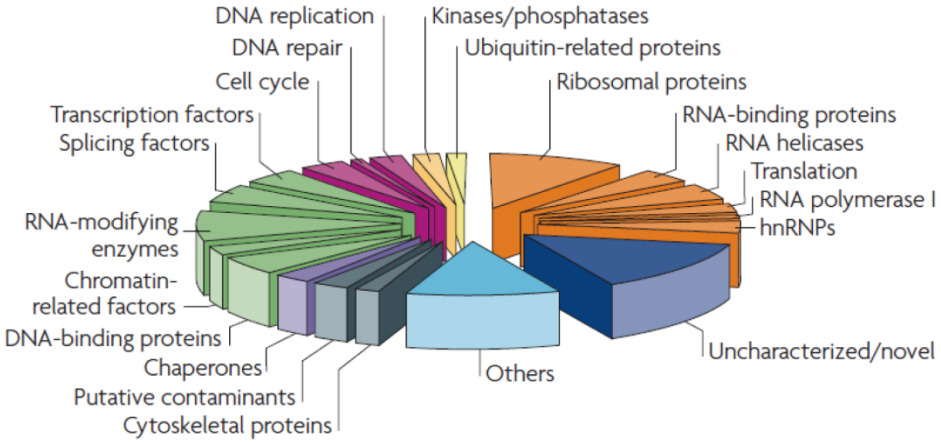


Figure 1.4 The nucleolar proteome. Figure shows the human nucleolar proteins identified by purification and mass-spectrometric analysis, which also highlights the classified cellular functions of these nucleolar proteins and indicates potential novel functions of the nucleolus. Figures from Boisvert et.al, 2007.

1.2.1 Nucleolus and cell cycle progression

One particular area regulated by nucleoli is the cell cycle. Firstly, the cell cycle itself influences nucleolar function. For example, factors in the Pol I transcription complex are modulated by different cyclin-dependent-kinases to dynamically activate or de-activate rDNA transcription and then subsequently assemble or dissasemble nucleoli. Secondly, as cells need nucleoli to produce sufficient ribosomes for protein-synthesis, cell growth and division is tightly linked with the nucleolus. It is no doubt that cells with impaired nucleoli and limited ribosome production will be disfavoured by natural selection and destined to die. It has been well established that perturbation of ribosome synthesis can cause cell cycle arrest through deprivation of newly synthesised ribosomes (Tsai and Pederson, 2014). However, beyond this obligatory relationship, nucleolus has also been shown playing roles in coordinating cell-cycle-control in ways that unrelated to its ribosome production function. This was first mentioned by the Thoru Pederson lab, as they selectively inhibited rDNA transcription using low dose of actinomycin D, which had minor effect on cells' ribosome store, had caused cell cycle arrest (Pederson and Powell, 2015). Then a nucleolar surveillance system centred around the p53 pathway has been proposed in the regulation of cell cycle progression after nucleolar disruption (Tsai and Pederson, 2014).

Probably, the most intensively studied mechanism linking nucleoli to cell cycle progression is activation of p53. p53 also participates in other downstream effects of nucleolar perturbation. How p53 contributes to the 'plurifunctional nucleolus' will be discussed in full below.

The nucleolus may also regulate the cell cycle by sequestering specific cell cycle proteins. One example is, in yeast, a protein phosphatase named cdc14 is sequestered in the nucleolus by the anchoring protein Net1 during interphase. Upon entry into anaphase, Net1 is phosphorylated, which releases activated cdc14, allowing it to dephosphorylate the mitotic cyclin degradation activator—Cdh1, thus promoting exit from mitosis. Nucleolus can also regulate cell cycle by merely sequestering factors required for cell cycle in this subcompartment without any post-translational modification process. One example is the nucleolar sequestration of telomerase. In primary human cells, the telomerase remains in the nucleolus until the beginning of telomeres replication at the late stage of S phase. This regulated sequestration-release has been suggested to play a crucial role in ensuring the appropriate timing of telomeres replication, as this sequestration is disrupted and telomerase mobilizes from nucleolus to nucleoplasm either in transformed cells or in cells underwent DNA damage (Wong et al., 2002).

1.2.2 Nucleolus and cell apoptosis, cellular senescence, stem cell biology and viral replication

Beyond cell cycle and cell growth control, studies have broadened the role of nucleoli to other fields like cell apoptosis, cellular senescence, stem cell biology and viral replication.

A number of evidences have revealed a role of the nucleolus in regulating programmed cell death. RelA is a transcription factor of the NF-κB signalling pathway, which binds to targeted gene promoter and drives gene expression in the nucleoplasm. In colorectal cancer cells, proapoptotic stimuli (such as aspirin treatment, UV-C exposure and serum deprivation) caused cell apoptosis through sequestration of transcription factor NF-κB RelA in the nucleolus and inhibiting NF-κB-driven pro-survival effects (Stark and Dunlop, 2005). Furthermore, in a parallel

way, nucleolar sequestration of RelA triggered translocation of nucleolar protein nucleophosmin (NPM) from nucleolus to cytoplasm, where it facilitated BAX mitochondrial accumulation and then cell apoptosis (Khandelwal et al., 2011). A recent study further supported the link between the nucleolus and BAX-mediated apoptosis. Peter Pan (PPAN), a protein mainly localized in the nucleolus and mitochondria, was released from nucleolus and accumulated in mitochondira under nucleolar stress, where it increased BAX-mediated mitochondrial outer membrane permeabilization and promoted cell apoptotic activity (Pfister et al., 2015).

There are few but provocative evidences link nucleolus to cellular senescence, the majority of which are focus on the human-aging-linked Werner Syndrome (WS), although the mechanism of this connection have been poorly investigated (Pederson, 2011). Briefly, the lack of an active Werner syndrome protein (WRN) would lead to WS, studies shown the human WRN displayed a nucleolar location, and identified amino acids 949-1092 are responsible for it (Marciniak et al., 1998; von Kobbe and Bohr, 2002).

The possible connection between the nucleolus and stem cell biology began to receive attention when, in a nuclear cloning experiment, the donor nucleolus was found experienced immediate reversible disassembly after a somatic nucleus injecting to the egg cytoplasm. It also found two Xenopus germ cell RNA-binding proteins FRGY2a and FRGY2b were responsible for this intriguing behaviour (Gonda et al., 2003). There were more links arose, that Martindill and his colleagues found phosphorylation status of nucleolar protein Hand 1 in trophoblast stem cell plays as a molecular switch from cell proliferation and renew to commitment giant-cell differentiation (Martindill et al., 2007).

There is evidence to suggest that RNA viruses interact with nucleoli to facilitate virus replication by consuming materials in the nucleolus (Hiscox, 2007). Studies also demonstrate nucleolar trafficking of regulatory factors is indispensable for HIV replication (Michienzi et al., 2006; Michienzi et al., 2002), Although the mechanism linking nucleoli and viral replication still unclear, it dose provoke an interesting question that why certain viruses specifically choose the nucleolus rather than other nuclear regions as a site of assembly (Pederson, 2010).

1.3 Nucleolus in disease and cancer

Given that ribosomal biogenesis is a tightly controlled process that plays a critical role in the maintenance of cellular homeostasis, metabolism, proliferation and growth, it is not surprising that disruption of it leads to pathophysiological disorders. Indeed, dysregulation of ribosomal proteins and Pol I transcription are linked with a broad range of human diseases.

Ribosomopathies are a heterogeneous class of diseases caused by genetic mutations and loss of functions in the molecular constituents of the ribosome. Examples of ribosomopathies include Diamond-blackfan anemia and 5q- syndrome. Other disorders intimately associated with rDNA transcription and processing include Treacher Collins Syndrome, Werner Syndromes, Shwachman–Diamond Syndrome and Cartilage Hair Hypoplasia (Narla and Ebert, 2010). It is still unclear how such mutations in ribosomal proteins result in rare congenital syndromes. However, there is increasing evidence to show nucleolar stress and activation of the HDM2-p53 surveillance pathway are critical for the pathological effects of these mutations (Ellis, 2014; Hannan et al., 2013; Nakhoul et al., 2014; Narla and Ebert, 2010).

In addition, gene mutations on factors associated with rDNA transcription could lead diseases by directly affecting Pol I transcription process. For example, Treacher Collins Syndrome (TCS) is a ribosomopathy that primarily results from a mutation in one copy of *TCOF1* gene. *TCOF1* encodes a phosphoprotein called treacle, which localizes to the DFC of the nucleolus and associates with pre-rRNP complex. Treacle regulates Pol I transcription by directly interacting with rDNA chromatin, the Pol I enzyme and UBF. It was shown that *TCOF1* gene knock out in mouse embryos caused similar growth retardation and craniofacial abnormalities as human TCS (Valdez et al., 2004). Filamin A is a well-characterised F-actin binding protein acting as a signalling scaffold to regulate cell shape and mobility. Filamin A is encoded from the *FLAN* gene, mutation of which has been linked to a number of human genetic disorders with skeletal, craniofacial or cardiovascular defects (Hannan et al., 2013). One study has demonstrated Filamin A localizes to nucleolus and associates with the Pol I enzyme and the Pol I complex component, TIF-IA (Rrn3 in mouse).

This interaction prevents Pol I binding the rDNA promoter and thus, inhibits rDNA transcription (Deng et al., 2012).

In addition to ribosomopathies, a wide range of growth dependent and developmental diseases are associated with dysfuntion of Pol I transcription, directly or through dysregulated upstream signalling networks. For example, diseases like muscle hypertrophy and atrophy originate from the imbalance between protein synthesis and degradation at the cellular level. A wide range of studies have demonstrated that a central mechanism for the development of these diseases is the dysregulation of growth related signalling pathways, such as PI3K/AKT/mTOR and RAS/RAF/ERK, and the subsequent modifications of key modulators of Pol I transcription (Hannan et al., 2013). Nucleolar stress is also associated with the pathogenesis of neurological diseases, for example, Parkinson's disease and Huntington's diseases (HD) (Lee et al., 2014; Parlato and Liss, 2014). In respect to the molecular mechanisms, studies have shown dysfunction of crucial Pol I factors can lead to neurological disorders. For example, post-translational modifications on UBF, such as acetylation and methylation, have been shown to play a central role in the development and progression of HD (Lee et al., 2014); Neuronal degeneration is promoted in *TIF-IA* gene knock-out mice or in neurons with perturbed TIF-IA(Parlato et al., 2008; Rieker et al., 2011).

1.3.1 Nucleolus and cancer

Impairment of nucleolar functions is also an indicator of cancer development and emerging as a promising therapeutic target for cancer treatment. Firstly, increased Pol I transcription leads to enlargement of nucleoli. Indeed, abnormal nucleolar size has been used by pathologists as an indicator of tumour proliferation as much as a century ago, well before the primary function of nucleoli was established. Secondly, tumour cells need hyper-activated Pol I transcriptional activity to meet the high protein needs required for uncontrolled proliferation. Generally, tumour cells abnormally promote Pol I transcription by means of hyper-activate oncogenes, inactivate tumor suppressors and up-regulate protein kinase. For example, oncogene c-Myc enhances the association of SL1/TIF-IB to rDNA promoter or directly up-regulates expression of UBF (Schlosser et al., 2003), and a murine model of

spontaneous lymphoma overexpressing MYC in B lymphocyte (Eμ-Myc) displayed highly expression of TIF-IA and UBF compared to normal B lymphocytes (Bywater et al., 2012). This indicates the increased Pol I transcription is an early event in carcinogenesis. Therefore, suppression of hyperactivated rRNA production is a good approach to restrain the uncontrolled cell proliferation of tumour cells.

1.3.2 Targeting Pol I factors for disease therapy

Targeting the nucleolus and ribosome biogenesis for disease therapy remains controversial. As ribosome biogenesis is essential to maintain proliferation of normal cells, it is technically difficult to design drugs that can act specifically on diseased cells with impaired nucleoli and ribosome biosynthesis (Quin et al., 2014). However, as ribosome biosynthesis is regulated by numerous processes, many clinically approved drugs have been shown to alter their activities, at least in part, through affecting Pol I transcription. A screen of current commonly used chemotherapeutic agents found that the majority of these drugs (21 out of 36) could affect ribosome biogenesis, although at different levels and stages (Burger et al., 2010). Actinomycin D and doxorubicin intercalate with rDNA to inhibit Pol I transcription. 5-fluorouracil affects the late rRNA processing through incorporating into the 47s pre-rRNA. Cisplatin crosslinks rDNA and inhibits Pol I transcription by possessing a high affinity with UBF thus hijacking it away from the place of action, this is extremely interesting as there is a strong inverse correlation between UBF expression level and Cisplatin sensitivity in patients (Huang et al., 2002); Notably, a most recent study found the platinum analogs of cisplatin, carboplatin and oxaliplatin, result in cell death through inducing ribosome biogenesis stress. Oxaliplatin, which is frequently applied in colorectal cancer treatment, enhance the nucleolar foci formation in a similar way as actinomycin D. In addition, the study also demonstrated the nucleolar stress response pathway ‘RPL11-MDM2-P53’ was essential for oxaliplatin cytotoxicity (Bruno et al., 2017).

Although many commercialized therapeutics act indirectly on rRNA biogenesis, a number of small molecule inhibitors have emerged that specifically target Pol I transcription. CX-3543 (also called quarfloxin) was the first of these. CX-3543 disrupts the nucleolin protein/G-quadruplex DNA complex in rDNA, a critical

interaction for Pol I transcription that is particularly amplified in cancer cells. By acting in this way, the small molecule inhibits Pol I transcription and induces tumour cells apoptosis (Drygin et al., 2009). CX-3543 had been carried through to phase II clinical trial on patients with carcinoid and neuroendocrine tumours, based on the potential clinical benefits from the scientific and clinical findings of a phase I trial in 2009 (Drygin et al., 2010).

More recently, two novel Pol I transcription inhibitors, CX5461 and BMH-21, were developed to selectively target tumours cells while spare normal cells. CX5461 potently and selectively inhibits Pol I transcription by preventing SL1/TIF-IB bind to the rDNA promoter and impairing the initiation stage of rDNA transcription. The downstream consequences of CX5461 treatment are cell senescence and autophagy (Drygin et al., 2011). Further investigation into the tumour specific activity of CX5461 suggested heamatologic malignancies are more sensitive to CX-5461 than solid tumours. This is an intriguing finding and led to questions about the molecular mechanism underlying this selectivity. The same group also found CX-5461 has very limited toxicity on normal B cells (Bywater et al., 2012). One hypothesis is that a p53 dependent pathway is central for predicting sensitivity to CX-5461 (Drygin et al., 2014). This hypothesis came from evidence demonstrating CX-5461-induced apoptosis in lymphoma cells is dependent on activation of a functional p53 pathway, while in normal B cells p53 stays inactivate in response to CX-5461 treatment (Bywater et al., 2012). In addition, sensitivity to CX-5461 in solid tumours is variable and p53-independent (Drygin et al., 2011). This hypothesis could be supported by a more recent study showing there is a direct relationship between the amount of stabilized p53, cellular ribosome biosynthesis rate and ribosome inhibition outcome (Scala et al., 2016). Interestingly, CX-5461 induced p53-dependent apoptosis is rapid but does not interfere with further ribosome production or protein translation, and this can even be achieved by transient treatment of the agent (Negi and Brown, 2015). Given the significant cancer preventative effect of CX-5461, it is now tested in clinical trial for patients with advanced haematological malignancies (Hannan et al., 2013).

BMH-21 is another small molecule that represses Pol I transcription and that has anti-tumor activity. It acts by a combination of specifically binding to GC rich regions on the rDNA promoter and mediating proteasome-dependent degradation of Pol I enzyme subunit RPA194 (Peltonen et al., 2014). One study demonstrated BMH-21 exerts anticancer activity without triggering DNA damage responses, which make it a promising therapeutic agent with a low cost of normal tissue toxicity (Colis et al., 2014)

Drug	Mechanism of action	Impact on ribosome biogenesis	Impact on nucleolus	Cancer type(s)
Mitomycin C	Interstrand DNA crosslinking via alkylating 5-CpG-3 guanosine	Inhibition of Pol I transcription	Nucleolar disintegration	Adenocarcinoma, stomach, pancreas, anal, bladder, breast, cervical, colorectal, head, neck, and non-small cell lung cancer
Cisplatin	DNA crosslinking via alkylating DNA bases	Inhibition of Pol I transcription	Nucleolar disintegration	Testicular, bladder, lung, oesophagus, stomach, ovarian sarcoma, and lymphoma
Oxaliplatin	DNA crosslinking via alkylating DNA bases	Inhibition of Pol I transcription	Nucleolar disintegration	Oesophagus, stomach cancer, and colorectal carcinoma
Mitoxantrone	Topoisomerase II inhibitor and intercalates into DNA	Inhibition of Pol I transcription	Nucleolar disintegration	Breast, prostate, liver cancer, myeloid leukaemia, and non-Hodgkin's lymphoma
Doxorubicin	Intercalates into DNA and inhibits Topoisomerase II	Inhibition of Pol I transcription	Nucleolar disintegration	Bladder, breast, stomach, lung, ovarian, and thyroid cancer, leukaemia, Hodgkin's lymphoma, myeloma
Camptothecin (Topotecan and Irinotecan)	Inhibits Topoisomerase I	Modulates early rRNA processing	Nucleolar disintegration	Ovarian, lung, colon, and cervical cancer
Temsirolimus Everolimus	mTOR inhibitors	Inhibition of Pol I transcription	No effect	Renal cell carcinoma, progressive neuroendocrine tumours of pancreatic origin (PNET), subependymal giant cell astrocytoma (SEGA) associated with tuberous sclerosis (TS), and hormone receptor-positive, HER2-negative breast cancer in combination with exemestane
5-Fluorouracil	Thymidylate synthase, incorporates into 47S pre-rRNA	Impairs late rRNA processing	No effect	Colon, oesophageal, gastric, rectum, breast, biliary tract, stomach, head and neck, cervical, pancreas, renal cell, and carcinoid cancer
Homoharringtonine	Translation inhibitor, prevents elongation	Impairs late rRNA processing	No effect	Chronic myelogenous leukaemia (CML)
Actinomycin D	Intercalates into GC-rich duplex DNA	Inhibits Pol I transcription at low nanomolar concentrations	Nucleolar disintegration	Wilms tumour and Ewing sarcoma
CX-3543	Disrupts nucleolin/ rDNA G-quadruplex complexes	Selective inhibition of Pol I transcription (elongation)	Redistribution of nucleolin, no effect on fibrillarin	Phase I clinical trial Carcinoid and neuroendocrine tumours
CX-5461	Inhibits SL-1 preinitiation complex formation at the rDNA promoter	Selective inhibition of Pol I transcription (initiation)	Nucleolar disintegration	Phase I clinical trial AML, multiple myeloma and lymphoma
Ellipticine	Impairs SL-1 rDNA promoter binding and preinitiation complex assembly	Pol I transcription (initiation)	Not determined	Phase I and II clinical trial

Table 1.2 Chemotherapeutics inhibit ribosomal DNA transcription, which therefore contribute to their anti-cancer activity. Table from Hein et.al, 2013.

1.4 Nucleolus under stress

Given the energy required in the generation of ribosomes, it is not surprising that there is a close link between ribosome biogenesis and cellular homeostasis. Indeed, any environmental or internal stress that threatens homeostasis inhibits ribosome biogenesis and causes stress response on nucleolus.

In stressed cells, changes in ribosome subunit production are always accompanied by distinct changes in the nucleolar architecture (Boulon et al., 2010). For example, DNA damage triggered by UV irradiation or DNA damaging drugs causes nucleolar segregation, which is segregation of the DFC and FC to the periphery of the nucleolus forming 'nucleolar caps' (Al-Baker et al., 2004; Rubbi and Milner, 2003); Inhibition of RNA Pol I machinery by Actinomycin D induces rapid shrinkage of nucleoli, formation of 'nucleolar caps' and repositioning of nucleolar and Cajal Bodie' proteins (Shav-Tal et al., 2005). In contrast, Inhibition of RNA Pol II by DRB induces unravelling and fragmentation of nucleoli (Rubbi and Milner, 2003); Viral infection can also cause modulation of nucleolar shape (Hiscox, 2007).

The nucleolar proteome also changes dynamically under stress conditions. It has been interestingly found that, under metabolic stresses like transcription inhibition and proteasome inhibition, and DNA damage inducing stresses like UV-C and ionizing radiation, nucleolar-associated proteins dynamically translocate between different cell compartments based on mass-spectrometry (MS)-based SILAC quantitative proteomics (Andersen et al., 2005; Boisvert et al., 2010; Moore et al., 2011). These discoveries reinforced the notion that nucleolus is a dynamic compartment. That is, in response to stress, nucleolus can act as a specific place where proteins can be temporarily sequestered to or excluded from, in order to participate certain signal transduction processes in their proper location, or just simply stabilize or destabilize them by separating them from their interacting partners. This notion further makes it interesting to understand nucleolar proteins associated signaling pathways under stress conditions.

1.4.1 p53-dependent pathways

Although it is well established that nucleolar architecture and the nucleolar proteome are altered in response to stress, and that nucleoli coordinate stress response, the molecular mechanisms by which nucleoli sense stress and regulate the downstream consequences remain poorly understood.

The most studied downstream effector of nucleolar stress response is p53 (Figure 1.5). p53 protein, encoded from *TP53* gene, is well known as a tumor suppressor as it activates genes transcription or coordinates signalling pathways that results in inhibiting cell cycle progression, promoting apoptosis and autophagy. *TP53* gene is frequently mutated and recognized as a common feature of human cancers (Bouaoun et al., 2016). The functional properties of *TP53* gene mutations have been extensively investigated, establishing the essential role of p53 in cancer biology (Muller and Vousden, 2013).

Under unstressed condition, the level of active p53 is kept low due to MDM2 (murine/or HDM2 in human)-mediated p53 ubiqutinylation followed by nuclear export and degradation. MDM2 protein contains a N-terminus p53 binding domain, a C-terminus RING finger domain with E3 ubiquitin ligase activity and a central acidic domain which is likely to fold to make N-terminus bound p53 adjacent to carboxy RING finger for ubiquitin-mediated proteasomal degradation (James et al., 2014). One hypothesis is reducing the folding flexibility of MDM2's central acidic domain would prevent the ubiquitination and degradation of MDM2 bound p53, and it has been revealed that most of the stimuli or stress which cause activation of p53 pathway targeted this domain to block the activity of MDM2 (James et al., 2014).

p53 becomes activated by a set of protein kinases in response to a variety of stress, including but not limited to DNA damage, oxidative stress and aberrant oncogene expression. Interestingly, these stresses also cause nucleolar disruptions, implies that a common denominatior of stresses that stabilise p53 is disruption of nucleoli (Rubbi and Milner, 2003). The link between nucleolar disruption and stabilization of p53 was first evidenced by Rubbi and Milner. They demonstrated that micropore irradiation-induced DNA damage was unable to stabilize p53 unless the nucleolar

disruption was concurrently occurred. In addition, directly impaired nucleolar function by blocking the upstream binding factor (UBF) stabilizes p53 without causing DNA damage (Rubbi and Milner, 2003). A later study strongly supported this model by finding TIF-IA knock-out, which lead to nucleolar disruption, also induced cell death and upregulation of p53 levels in MEFs (Yuan et al., 2005).

Since then a huge amount of literature has described the molecular pathways by which disruption of the nucleolus stabilises p53. Upon nucleolar stress, the defects in ribosome biosynthesis are always coupled with aberrant redistribution of ribosomal proteins (RPs). Several ribosomal proteins (e.g RPL11, RPL5, RPL23, RPS3, and RPS7) have been shown to bind MDM2, thus stabilising p53 (James et al., 2014). RPL11, RPL5 and 5s rRNA can also form a trimeric complex to bind MDM2 and block the uquitin-mediated p53 degradation (Donati et al., 2013; Sloan et al., 2013). Based on this and many coherent studies, Deisenroth et.al proposed a 'RP-MDM2-p53 ribosome biogenesis surveillance pathway'. They suggested that in response to unfavourable stress conditions, disruption in rRNA synthesis/processing, or RPs imbalances, would allow a subset of RPs to bind to MDM2, thus inhibiting its interaction with p53, evoking cell arrest or apoptosis (Deisenroth and Zhang, 2010). In this way, nucleolar stress response also acts as a surveillance mechanism that inspects the quantitiy and quality of ribosomes. This surveillance pathway has also been shown in response to oncogenic stress specifically induced by oncogene c-Myc. Study found MDM2 carrying a cysteine-to-phenylalanine mutation in the acidic domain has impaired binding ability to RPL5 and RPL11. Mice harboured this mutation lost the p53 protective action in response to ribosome biosynthesis perturbation and accelerated c-Myc-induced lymphomagenesis (Macias et al., 2010).

Interestingly, in a reverse way, p53 was also shown necessary for the initial release of ribosomal proteins from nucleolus under DNA damage stress. In a quantitative proteomic study using unbiased MS-based SILAC, FM. Boisvert and A.I. Lamond found in p53-wild type cells, nucleolus exhibited specific segregation and ribosomal proteins like RPL11 exported from nucleolus following etoposide treatment. In comparison, p53-knockout cells showed less extent of nucleolar segregation and had less effect on ribosomal proteins nucleolar localization under the same condition.

This study indicates a feedback regulation between p53 and ribosomal proteins under stress condition (Boisvert and Lamond, 2010).

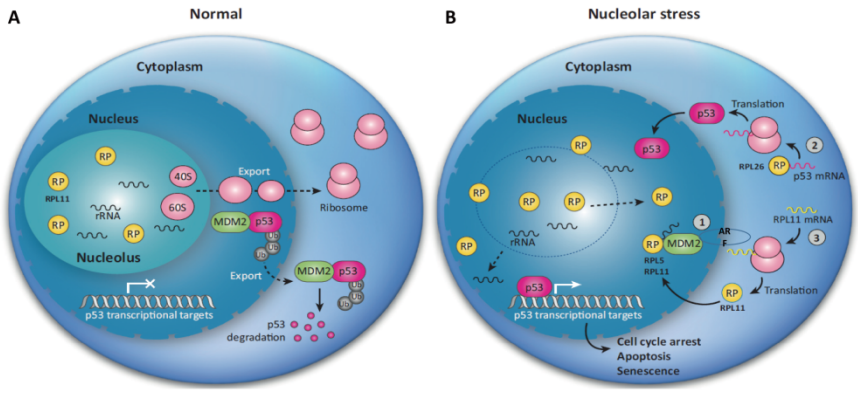


Figure 1.5 p53-dependent nucleolar stress response. (A) shows, in normal condition, MDM2-mediated p53 degradation keeps cellular p53 in a low level. (B) shows nucleolar stress triggers various modulations on ribosomal proteins (RPs) or nucleolar proteins, like p14ARF, that disrupt MDM2-p53 interaction, therefore stabilising p53 and causing cell cycle arrest or cell apoptosis. Figure adapted from Hein et.al, 2013.

Another nucleolar factor known to regulate MDM2 activity and p53 stabilization in response to stress is p14ARF. p14ARF (p19ARF in mice) is one of the alternate reading frame protein products of the *ARF-INK4a* locus. It functions as a tumour suppressor mainly through its ability to bind with MDM2 thus activating p53. In addition, p14ARF can directly modulate rRNA biogenesis through interfering with other nucleolar factors, such as UBF (Ayrault et al., 2006), or binding to 5.8S rRNA (Sugimoto et al., 2003).

In non-stressed cells, p14ARF is maintained at low levels in the nucleolus. Following oncogenic stress, such as Myc and Ras overexpression, p14ARF is upregulated and released from nucleoli. This allows it to bind to the central acidic region of MDM2, which prevents degradation of p53 and thus, controlling cell growth through cell cycle arrest or inducing apoptosis (James et al., 2014). The central importance of the p14ARF-MDM2-p53 pathway is highlighted by the finding that tumours often display mutated or silenced p14ARF (Sherr, 1998). Activity and stabilization of p14ARF is mainly regulated by its interaction with nucleophosmin (NPM), another nucleolar stress regulator. Studies have demonstrated NPM interacts with p14ARF to prevent ubiquitin-mediated degradation of p14ARF (Chen et al., 2010). NPM is also up-regulated by oncogenic stress therefore stabilizing p14ARF and promoting the

interaction between p14ARF and MDM2 and activating p53. However, there is a negative feedback loop in this pathway, as evidenced by overexpression of p14ARF promotes the ubiquitination and degradation of NPM, which subsequently disrupts ribosomal biosynthesis (Itahana et al., 2003).

1.4.2 p53-independent pathways

However, given the following three facts: 1. Nucleoli in yeast response to stress as human cells but there is no characterized human p53 or MDM2 homologues in yeast; 2. More than 50% of known cancer cells have non-functional p53; 3. The huge changes in the nucleolar proteome in response to stress, it is likely that other p53-independent signalling pathways are affected and involved in the downstream consequences of nucleolar stress (James et al., 2014). Clarifying these pathways would benefit for targeting p53-deficient cancers. However, current knowledge regards this are extremely deficient.

In recent years, there are emerging evidence have demonstrated nucleolar stress directly triggers additional signaling bypassing p53 pathway, and results in regulation of cell cycle process, cell growth and cell death. These downstream effectors of nucleolar stress include factors, such as E2F1, c-Myc and NF-κB, which have been known to be critical in controlling diverse cell functions.

E2F1 (E2F transcription factor 1) is a crucial transcription factor that controls expression of genes favoring cell cycle progression and cell proliferation, and is highly expressed in many types of cancer. Its transcriptional activity is negatively controlled by interacting with retinoblastoma protein pRB and is modulated by the upstream cyclin-dependent kinase, such as cyclin D1/CDK4. In another circumstance, it also plays an important role in mediating both p53 dependent and independent apoptosis mainly through up-regulating various pro-apoptotic genes. Notably, it has been shown down-regulation of E2F1 links ribosome biogenesis perturbation and cell cycle arrest in cells with inactivated p53. Donati et.al discerned the E2F1 involved nucleolar stress response pathway, that upon rDNA suppression by selectively depletion of a subunit of Pol I POLRIA, the release of ribosomal protein RPL11 antagonizes the interaction between E2F1 and MDM2 and leads to

destabilisation of E2F1, resulting in G1 cell cycle arrest (Donati et al., 2011). This finding indicates a potential role of E2F1 as a downstream effector of nucleolar stress.

c-Myc is a strong proto-oncogene that is mutated, deregulated and constitutively overexpressed in many cancers. Transcription factor c-Myc has been demonstrated critical in controlling cell proliferation and cell differentiation through facilitating expression of a large cohort of factors, including those involved in crucial steps of rRNA synthesis and ribosome production. Studies have implied c-Myc could potentially be a downstream effector of nucleolar stress based on the finding that ribosomal proteins, particularly RPL11, negatively regulate the transcriptional activity of c-Myc. This has been supported by the finding revealed ribosomal stress stimuli, like low dose actinomycin D and 5-fluorouracil, enhanced the association between RPL11 and the 3'-untranslatied region (3'-UTR) of c-Myc mRNA thus down-regulating the expression of c-Myc (Challagundla et al., 2011).

Most recently, another p53-independent nucleolar stress response pathway has been defined and involves Peter Pan (PPAN). PPAN is known to be involved in mRNA splicing and ribosomal maturation. The endogenous PPAN is mainly localized in nucleoli and mitochondria. Upon nucleolar stress stimuli, such as staurosporine and actinomycin D, PPAN released from nucleoli to cytoplasm where it was cleaved and degraded in a caspases-dependent pathway. The cytoplasmic translocation and loss of PPAN under stress condition subsequently caused destabilization of nucleolar factor UBF and NPM, and increased BAX-mediated mitochondrial outer membrane permeabilization thus triggering cell apoptosis. They also shown this novel nucleolar stress response pathway is through a p53-independenet fashion (Pfister et al., 2015). This study indicates a role of PPAN as a novel nucleolar stress effector, which links UBF, NPM and mitochondrial-mediated cell apoptotic pathway.

1.5 NF-kappaB

Similar to the p53 pathway, NF-κB plays a critical role in maintaining cellular homeostasis in response to stress, is dysregulated in cancer and controls cell proliferation and apoptosis. Emerging data also shows NF-κB as a candidate effector of nucleolar stress response. Studies from the Perkins' lab revealed the NF-κB

pathway is suppressed by the nucleolar protein p14ARF through impairing the transcription activation domain of RelA in response to oncogenic activation (Rocha et al., 2003) (Rocha et al., 2005). There are also other proteins that are known to be involved in the NF-κB response to stress reside within nucleoli, such as CK2 and Eukaryotic translation initiation factor 2α (EIF2α) (Jiang et al., 2003; Kato et al., 2003). In addition, there is a growing body of literature indicates most of the stresses that disrupt the nucleolus can activate the NF-κB signalling pathway. In this section I will introduce the NF-κB signalling pathway, mainly focussing on modulation of this pathway in cancer and under stress condition.

The NF-κB transcription factor is a ubiquitously expressed family of proteins that play a critical role in multiple physiological processes including immune and inflammatory response, cell growth and proliferation, cell differentiation, apoptosis and stress response. In mammalian cells, the NF-κB transcription factor family consists of five members: NF-κB 1(p105/p50), NF-κB 2(p100/p52), RelA (p65), RelB and c-Rel. Among them, NF-κB1 and NF-κB2 are initially transcribed as precursor forms p105 and p100, which, in the process of activation, are cleaved to form the mature proteins p52 and p50 (Hayden and Ghosh, 2012). All NF-κB family members share a N-terminal Rel homology domain (RHD), which supports their function of subunit dimerization and DNA binding. A nuclear localization sequence (NLS) resides within the RHD domain that facilitates their nuclear translocation. In resting state, the NLS on NF-κB family proteins is bound by the multiple ankyrin repeat domains (ANK) on a group of NF-κB inhibitor, IκB family (IκBα, IκBβ, IκBε, IκBγ, Bcl-3, IκBNS, p100 and p105). This binding enables IκB family halt the function of NLS and contribute to the sequestration of NF-κB proteins in the cytoplasmic of unstimulated cells.

NF-κB family proteins form homodimer or heterodimer which bind to κB consensus sequences on the promoters and enhancers of target genes. This can both positively and negatively regulate gene transcription dependent on the types of dimers (Chen et al., 1998). Only RelA, RelB and c-Rel contain a C-terminal transactivation domain (TAD), which confers their ability to initiate transcription. p52 and p50 lack this domain and so, p52 or p50 homodimers always act as transcription suppressors,

competing with TAD-containing heterodimers for binding to κB sites (Hayden and Ghosh, 2012). Once in the nucleus, the strength and kinetics of NF-κB driven transcription is influenced by a number of factors including binding of co-activators and repressors (e.g P300/CBP, p53 HDACs) and post-translational modifications (PTMs) of NF-κB proteins (Hayden and Ghosh, 2012).

NF-κB is the most central factor that regulates both innate and adaptive immune response, in the past 30 years, huge amount of works has been done to elucidate the role of NF-κB as a master regulator in inflammatory and immune response and results in a well-defined and complex signalling pathways network.

Dependent on the stimulus, NF-κB signalling can be activated by the "canonical" or "non-canonical" pathways (Gerondakis et al., 2014). The two pathways differ in the upstream activation cascades and the NF-κB family members involved as well as the eventual outcome on gene expression and biological functions (Figure 1.6). The NF-κB canonical pathway, or classical pathway, represents the general scheme of NF-κB pathway regulation and is very well established. Upon exposure of cells to stimuli such as cytokines, cellular stress or infection, triggered signals culminate in the phosphorylation and activation of the IκB kinase (IKK) complex, which is composed of the two kinase subunits IKKα and IKKβ, and one regulatory protein NEMO (or IKKγ) (Hinz and Scheidereit, 2014). Activated IKKs, especially IKKβ, phosphorylates IκBα on serines 32 and 36 and targets IκBα for ubiquitination and degradation by proteasome, allowing dimers (mainly RelA/p50) to translocate to the nucleus, bind to the specific κB motif and regulate gene expression. The non-canonical NF-κB pathway is induced by specific stimuli, such as CD40 ligand, B-Cell activation factor (BAFF), receptor activator of nuclear factor κB ligand (RANKL) or lymphotoxin-β. It is dependent on NF-κB-inducing kinase (NIK)-induced phosphorylation of IKKα and subsequently phosphorylation and cleavage of p100, which leads to formation and nuclear translocation of a RelB/p52 complex (Sun, 2011). As almost all research to date suggest stresses, chemopreventative and chemotherapeutic agents regulate the canonical pathway, only this will be further discussed in detail.

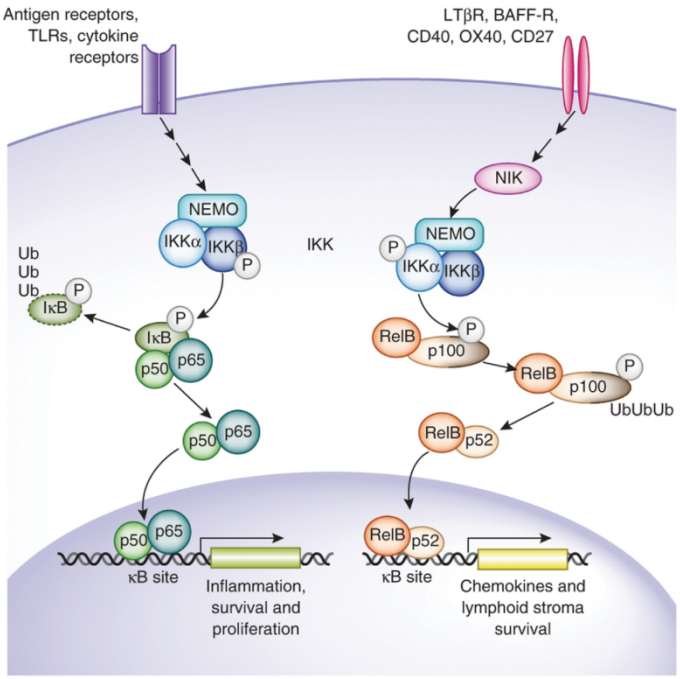


Figure 1.6 The NF-κB signalling pathway. A schematic diagram shows the canonical and non-canonical NF-κB signalling pathway. Figure from Gerondakis et al., 2014.

1.5.1 NF-κB in cancer

NF-κB is crucial for human health and aberrant regulation of nuclear NF-κB activity is always associated with development of disorders, (e.g inflammatory bowel disease, autoimmune disorders, and cancer (DiDonato et al., 2012)). The first line of evidence suggesting an association between NF-κB and cancer came from studies that show the genes encoding NF-κB transcription factor family members, IκB and IKK proteins are mutated, deleted or chromosomally translocated in lymphoid malignancies (Xia et al., 2014).

Although in some specific cases NF-κB seems to be anti-tumourigenic, such as in Ras-induced invasive growth of epidermal tumours and in carcinogen-induced hepatocellular carcinomas, the constitutive activation of NF-κB is prevalent in most major types of cancers and is believed to be an important driver of tumorigenesis (Ben-Neriah and Karin, 2011). Multiple mechanisms have been proposed to explain how NF-κB is constitutively activated in tumours and how this activation contributes to carcinogenesis (Figure 1.7).

It has been proposed that NF-κB constitutive active in tumours may be the result of two events: gene mutations that affect pathway components and/or increased inflammatory cytokines in the tumour microenvironment which chronically activate NF-κB (Ben-Neriah and Karin, 2011). Gene mutations that activate NF-κB signalling were first evidenced in MALT lymphomas (a group of tumours involving the mucosa-associated lymphoid tissue). It was found that rearrangement of Bcl-10 and MALT1, which were common mutations in MALT lymphomas, lead to assembly of a protein complex that activated IKKs and NF-κB (Uren et al., 2000). Another mutation on Toll-like receptor (TLR) adaptor, MYD88, has also been found to activate NF-κB and contribute to diffuse large B cell lymphoma. The L265P mutation in the MYD88 Toll/IL-1 receptor (TIR) domain promoted lymphoma cell survival by spontaneously assembling a protein complex containing MYD88 associated kinase, IRAK1 and IRAK4, enhancing IRAK4 kinase activity and phosphorylation of IRAK1, which in turn activated NF-κB (Ngo et al., 2011).

Constitutive activation of NF-κB in tumour cells can also arise from exposure to inflammatory cytokines in the tumour microenvironment. Chronic inflammation can promote cancer in multiple ways, such as prolonged activation of pro-proliferative pathways and generation of DNA damage that leads to oncogenic mutations (Grivennikov et al., 2010). As a central mediator of inflammatory response, NF-κB also acts as a master regulator of the cross-talk between chronic inflammation and carcinogenesis. This have been proven in inflammation-linked cancer models like colitis-associated colon cancer (CAC). A study in a mouse CAC model found that IKKβ in intestinal epithelial cells was activated and contributed to increased tumour incidence by inducting anti-apoptotic gene expression (Bcl-xL) and repressing pro-apoptotic gene expression (Bax and Bak). In contrast, conditional deletion of IKKβ in intestinal epithelial cells attenuated colitis-associated tumour incidence (Greten et al., 2004). In the same CAC model, conditional ablation of IKKβ in myeloid cells also decreased colitis-associated tumour incidence. Rather than affecting cell apoptosis, perturbation of IKKβ inhibited NF-κB mediated transcriptional activation of tumour-promoting paracrine inflammatory factors thus halting cell growth (Greten et al., 2004). These studies demonstrate that in inflammation associated cancer, activation of the NF-κB pathway can convey a pro-tumorigenesis effect both by

directly perturbing apoptotic-related gene expression in tumours cells and by indirectly affecting myeloid cells and hence, the production of tumour-promoting inflammatory factors. In agree with the findings in CAC model, additional studies in hepatitis-associated liver cancer, gastric cancer and lung cancer model also demonstrated that activation of NF-κB mediated inflammatory response is crucial for carcinomas.

Beside stimulation by inflammatory factors in the microenvironment, NF-κB in tumour cells can be triggered by the DNA damage/genomic instability associated with prolonged exposure to inflammatory cytokines. For example, ROS induced DNA damage, which will be discussed in the NF-κB under stress section (1.5.2).

Once activated, NF-κB can promote cancer initiation and development through multiple mechanisms including stimulation of cell proliferation/inhibition of apoptosis, regulation of tumour angiogenesis, promotion of tumour metastasis, facilitation of escape from immunosurveillance and remodelling of tumour metabolism (Perkins, 2012; Xia et al., 2014) (Figure 1.7). Importantly, it should be noted that all of these mechanisms modulated by NF-κB have been identified as hallmarks of cancer (Hanahan and Weinberg, 2011).

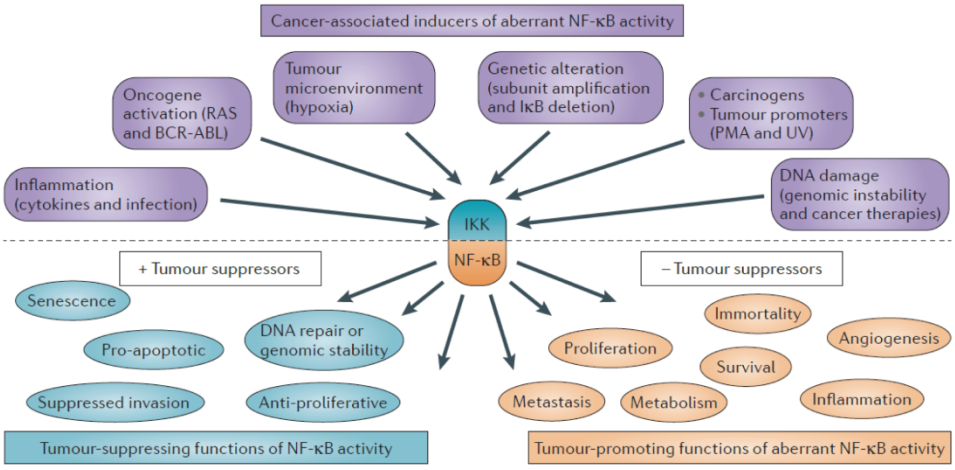


Figure 1.7 The causes and consequences of NF-κB activation in cancer cells. Gene mutations that affect components and pathways which subsequently activate NF-κB pathway and increased inflammatory cytokines in tumour microenvironment are two major events that contribute to constitutive activation of NF-κB in cancer cells. As consequences of NF-κB activation, cancer initiation and development are promoted through affecting hallmarks of cancer. Figure adapted from Perkins, 2012.

Among them, NF-κB had been well known to regulate cell proliferation and apoptosis mainly through its role as a transcription factor. As early as 18 years ago, study had demonstrated NF-κB contributes to cell growth and proliferation by regulating transcription of the cell-cycle regulator, cyclin D1 (Hinz et al., 1999). Studies in the past years have extensively expaned this finding both *in vitro* and *in vivo,* and have revealed and established a growing list of cell proliferation/apoptosis-related genes that are actived by NF-κB, including genes with pro-growth (GM-CSF, M-CSF and VEGF-C) and anti-apoptotic functions (X-linked inhibitor of apoptosis (XIAP), Inhibitors of Apoptosis (IAPs), Fas ligand and BCL2 family proteins) (website resource: http://www.bu.edu/NF-κB/gene-resources/target-genes/, maintained by Thomas Gilmore's laboratory). Similarly, in the list of known NF-κB transcriptional targets, numerous other genes have been recognized to regulate a wide spectrum of cellular functions that would contribute to cancer development. This includes, but not limited to, agenogenic molecules (Vascular Endothelial Growth Factor (VEGF), IL-8 and Matrix metalloproteinases (MMPs)), and cell adhesion molecules, chemokines and transcription factors that involved in tumour metastasis (chemokines receptor CXCR4, Twist 1 and Snail).

Besides modulating hallmarks of cancer for cancer promotion and progrsssion, a recent study demonstrated the role of NF-κB in cancer stem cells, which shed a new insight of NF-κB contribution to tumourigenesis. Using an inducible intestinal epithelial cell (IEC)-restricted β-catenin constitutive activation mice model that recapitulate most of the events during the colon cancer initiation, Schwitalla S et.al found NF-κB activity was significately elevated in Wnt signalling activated IEC cells and proliferative crypt stem cells (cancer stem cells). Notably, they demonstrated NF-κB activation promotes colorectal tumourigenesis through dedifferentiating intestinal cells and endowing them with cancer-stem-cell-like properties by direct targeting Wnt signalling and modulating Wnt-dependent stem cell gene expression (Schwitalla et al., 2013).

1.5.1.1 Crosstalk between NF-κB and p53 in cancer

Upon activation, NF-κB consistently inter-talks with other transcription factors to coordinate gene expressions and regulate tumourigenesis. One of the well-

established coordinator of NF-κB in this regulation is p53. NF-κB effects on cancer cell growth are strongly dependent on the cellular status of p53 and there are multiple areas of crosstalk between the two pathways.

Firstly, an early study had shown that NF-κB and p53 inhibit each other's gene transcriptional activities. They revealed that interactions with transcriptional coactivator proteins p300 and CREB-binding protein (CBP) are essential for the activity of both NF-κB and p53. However, the limitation of p300/CBP cellular pools led to a competition between the two transcriptiona factors for this functional binding (Webster and Perkins, 1999).

We now clearly know that, the relation between NF-κB and p53 is intricate and shows variability dependent on different cellular conditions. Although there are some studies showing NF-κB activation is correlated with p53-meidated tumour cell apoptosis, the majorty of studies in the crosstalk between these two vital transcriptional factors have pointed that NF-κB functions in cancer development are always antagonized by wild-type p53. For example, Perkins et.al found that regulation of mitochondrial respiration by NF-κB is dependent on the status of p53. They demonstrated that p53 inhibits translocation of RelA to mitochondria by blocking an essential interaction between RelA and mitochondrial heat shock protein HSP, mortalin. Upon deletion of p53, RelA was transported into the mitochondria where it directly repressed mitochondrial gene expression (Johnson et al., 2011). Considering that reprogramm of tumour cell metabolism is emerging as another hallmark of cancer and mitochondrial respiration is a critical metabolic event that is required to maintain cellular homeostasis, this finding implied a strong dependence of NF-κB on p53 status in regulating tumour metabolism and energy production.

In addition, many studies have shown that loss of p53 or p53 mutant upregulates the activity of IKKα/β and NF-κB transcription activity. Especially, in p53 deficient or p53 mutant condition, activated NF-κB is likely to play more important role in tumour development. For example, activation of NF-κB is evidenced essential in maintaining tumour metabolism in p53-null tumour model. Oncogenic Ras-induced transformation of p53-null MEFs and consequent increase in aerobic glycolysis were abrogated when p65 was simultaneously deleted (Kawauchi et al., 2008).

Interestingly, using a mouse model of lung adenocarcinoma that harbours conditional loss-of-function p53, Meylan et.al revealed tumours from this type of mice have elevated NF-κB activity compared to tumours from mice with wide-type p53. Whereas, restoring p53 in the model mice abolished increased NF-κB activity. Moreover, they evidenced inhibition of NF-κB *in vivo* by infecting mice with letivirus that constitutively express IκB-SR (dominant-negative form of IκBa) has almost completely blocked the initiation and development of lung tumours. Therefore, this study further addressed the importance of the p53 status in NF-κB activation-mediated cancer development (Meylan et al., 2009). Furthermore, common mutant p53 (mutp53) isoforms in pancreatic cancer cells or colorectal cancer cells prolonged low dose TNF-α-induced NF-κB activation and subsequently IL-8 elevation, indicating a role of mutp53 in promoting chronic inflammation response. This was further supported by *in vivo* experiments, that mice harbouring germline mutp53 were highly susceptible to inflammation inducing agent dextran sodium sulfate (DSS)-induced chronic inflammation, tissue damage and colon carcinoma. Notably, the accumulation of mutp53 in the inflamed colon or carcinoma was concomitant with the activation of NF-κB (Cooks et al., 2013).

Furthermore, NF-κB pathway components that contribute to tumourigenesis, such as IKKβ, can also target the p53 pathway. It has been shown that p53 contains a consensus motif which is harboured by most of the IKKβ substrates, like IκBα. Upon doxorubicin treatment, IKKβ regulates p53 stability through phosphorylating p53 at serine 362 and serine 366, thus leading it binds to E3 ligase β-TrCP–Skp1-Cullin-F-box protein (SCF) complex and degraded by ubiquitination-dependent proteasome degradation. Besides, p53 degradation under this pathway is independent of MDM2 acitity. Notably, this regulation only happens under DNA damage stress, but not under TNF-α stimulus, suggesting this particular IKKβ-mediated p53 modification is a specific stress response, or a result of DNA damage-induced chronic inflammation environment (Xia et al., 2009).

Given the importance of p53 in cancer development and ubiquitous of the inter-talks between p53 and NF-κB pathway, clarifying the p53 status is necessary when investigating the contribution of NF-κB in particular cancers.

1.5.2 NF-κB and stress

In addition to its role in immune function and cancer development, NF-κB is a critical regulator of stress response. The NF-κB pathway is activated by a wide variety of extracellular stresses such as nutrient stress, DNA damaging agents, chemotherapeutic/preventative agents and oxidative stress. Intracellular stress, including ER protein overload, are also know to stimulate the pathway. However, compared to the well-established role of NF-κB in immune response and cancer development, the role of NF-κB signalling in stress response, especially the molecular basis underlying stress stimulation of NF-κB, remains poorly understood. One question that needs to be answered is how such a diverse range of stresses and agents can activate NF-κB.

Oxidative stress is defined as an increase of the intracellular concentration of reactive oxygen species (ROS) such as H_2O_2, superoxide (O_2-), or hydroxyl radical (·OH). Oxidative stress was shown to activate NF-κB pathway and was once proposed as a common messager converging stresses on NF-κB activation based on a set of findings (Schmidt et al., 1995). Firstly, it was shown that micromolar concentrations of H_2O_2 activate the NF-κB pathway in T cell and HeLa cells. Most importantly, this activation was blocked by using specific antioxidant compounds such as N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), and by overexpressing antioxidative enzymes that decreased the level of intracellular ROS (Meyer et al., 1993; Schreck et al., 1991). Secondly, several studies demonstrated that ROS is necessary for NF-κB activation induced by many other agents including TNFα, okadaic acid, cycloheximide, double-stranded RNA, calcium ionophore, active phorbol ester, interleukin-1, lipopolysaccharide, lectin, and CD28 surface receptor.

ROS regulates the NF-κB pathway through directly modulating NF-κB proteins or pathways upstream of NF-κB. For example, Jamaluddin et.al found ROS enabled TNFα-induced IL-8 transcription through inducing phosphorylation of RelA on serine 276 by cAMP dependent protein kinase A (PKAc), thus promoting the binding between RelA and CBP/300 to enhance gene expression (Jamaluddin et al., 2007). Takada et.al demonstrated that rather than induce the typical TNF-α-induced S32/36

IκBα phosphorylation and degradation, H_2O_2 induces NF-κB activation and nuclear translocation through Syk-mediated phosphorylation of IκBα on tyrosine 42 without degradation of this protein. This suggested a distinct IκBα modification and NF-κB activation mechanism under ROS stress condition. However, they failed to explain how Syk-mediated NF-κB nuclear translocation could happen without IκBα degradation in this situation (Takada et al., 2003). These evidences show a complex regulation of NF-κB by oxidative stress. However, the mechanisms by which ROS regulates NF-κB remain controversial.

Although ROS induced NF-κB activation was proposed as a common model of stress-stimulation of NF-κB, emerging studies have provided evidence against this notion. One example is stress inducers that cause **DNA-damage** activate NF-κB without producing ROS. Compared with ROS-related stimulation of NF-κB, the primary pathways by which DNA damage activates NF-κB are clearer. DNA damage response (DDR) in mammalian cells is well established and mediated by a family of phosphatidylinositol 3-kinase like proteins—ATM, ATR and DNA-PK (McCool and Miyamoto, 2012). DNA damage can be induced by various simuli, including UV light exposure, ionizing radiation and agents like camptothecin and bleomycin. Stein et.al was the first to demonstrate DNA damage, caused by UV exposure, activated the cytoplasmic NF-κB pathway (Stein et al., 1989). Although there are still many unknowns regarding the molecular mechanisms involved in DNA damage-induced NF-κB activation, in the past 30 years a clear ATM-NEMO-TAK1-IKK axis has been identified. That is, upon sensing DNA damage or double strand breaks (DSB), DSB-responsive ATM kinase modulates nuclear NEMO by different means of post-translational modifications, including SUMOylation, phosphorylation and monoubiquitination. It is followed by the activation of growth factor bactivated kinase-1 (TAK1) in the cytoplasm, which subsequently phosphorylates and activates IKKs (McCool and Miyamoto, 2012).

In addition, DNA damage also regulates NF-κB in other atypical pathways. For example, Kato et.al proposed an IKKs-independent NF-κB activation pathway in response to UV-C stress. They found UV-C exposure in HeLa cells induced phosphorylation and degradation of IκBα, which is dependent on p38 MAPK

mediated CK2 activation. In addition, activation of this pathway resulted in a protective effect of cells against low dose UV-C. Notably, in this regulation, stress-activated CK2 phosphorylated IκBα at a cluster of C-terminal sites, but not the N-terminal sites which targeted by IKKs, indicating a stress specific regulation pattern (Kato et al., 2003).

An overwhelming number of studies indicate that **chemotherapeutic and chemopreventative agents** act on cancer cells, either positively or negatively, by activating the NF-κB pathway. For example, NF-κB activation was found to contribute to doxorubicin induced neuroblastoma cell death (Bian et al., 2001). In human non-small cell lung carcinoma cells, etoposides were shown to activate NF-κB before the induction of apoptosis (Tabata et al., 2001). Since many chemotherapeutic agents deliver their cytotoxicity by exerting oxidative stress or DNA damage, the mechanisms underlying NF-κB activation by these agents may be shared with those induced by ROS and DNA-damage. Another group of agents that act through the NF-κB pathway are nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin. These agents primarily provide analgesic, antipyretic and anti-inflammatory effects. However, it has been shown that long-term administration of this group of drugs has favourable effects on reduction of cancer incidence and prevention of tumor progression in many solid tumor entities like colorectal cancer (Rothwell et al., 2011). This suggests targeting NF-κB activation under low-grade inflammation context could be a potential strategy for cancer prevention and therapy. The effects of NSAIDs on the NF-κB pathway will be discussed in full in next section of this chapter (see section 1.6.2.2).

Studies as far have shown a complex regulation of NF-κB under different stress conditions. However, the mechanisms by which stress regulates NF-κB still remain controversial and unclear, while many of the different outcomes on regulation of NF-κB observed between studies are probably due to the cell-specific response and different experiment settings. It will be with great interests to investigate whether there is a common denominator that could sense a range of stress signals and culminate in activation of NF-κB pathway, like stress-induced stabilization of p53 through nucleolar stress-mediated regulation on MDM2.

1.5.3 NF-κB as a therapeutic target

Given the importance of NF-κB in multiple pathologies, including cancer initiation and progression, chemical compounds that specifically inhibit this pathway have been extensively explored. However, unfortunately, hundreds of compounds, most of which were efficient in laboratory studies, have not shown significant efficiency in clinical trials for cancer therapy (Ben-Neriah and Karin, 2011; Xia et al., 2014). Since NF-κB signalling is critical for innate and adaptive immune response, immunosuppression is a major toxicity. In clinical trilas the inhibitors have shown low efficiency and tumours have been found to aquire resistance. One possible direction to improve the efficiency is choosing the appropriate timing of compounds treatment. That is, applying NF-κB inhibitors may be more desirable at the chronic inflammatory stage when immune factors mainly contribute to tumourigenesis, rather than at the later tumour phases when immune cells primarily target and eliminate transformed cells (Hoesel and Schmid, 2013).

Another promising direction for improved NF-κB inhibitors efficacy would be the combination NF-κB inhibition with other cancer therapies (Xia et al., 2014). This strategy is mainly based on the fact that NF-κB pathway is activated under numerous cellular stresses, which can be induced by many kinds of anti-cancer therapies such as radiotherapy and chemotherapies as discussed above. From this aspect, NF-κB inhibition and anti-oncogenic therapies could have some synergetic anti-tumour effects. For example, Wang et.al demonstrated inhibition of NF-κB via the adenoviral-mediated delivery of super-repressor form of IκBα into tumour cells enhanced the sensitivity of chemoresistant cells to chemotherapeutic compound CPT-11 (Wang et al., 1999). Similarly, Bortezomib, a proteasome inhibitor which blocks degradation of IκBα, overcame chemoresistance in colorectal tumour cells when combined with CPT-11 (Cusack et al., 2001). However, the efficacy of this strategy has not been completely verified *in vivo* and in clinical patients.

Stress stimulation of NF-κB is a potential mechanism of the acquisition of resistance to chemo- or radio-therapies induced apoptosis. Therefore, NF-κB activation could be at least a potent biomarker for optimized chemo- or radio-therapies. Some *in vitro* study has shed light on this possibility, such as a more recent study evidenced NF-κB

activation-mediated mesenchymal differentiation and enrichment of CD44 subpopulations enhanced the radiotherapy resistance in glioblastoma. For my knowledge, there are some ongoing clinical trials investigating the relationship between NF-κB activation and cancer-therapy outcomes. For examples, the trial works on NF-κB activation in response to treatment with external beam radiotherapy in adenocarcinoma of the rectum (NCT00280761), and the trial studies the significance of NF-κB as a biomarker to predict the benefits of the postoperative adjuvant chemotherapy in stage II/III gastric cancer patients (NCT01905969) (Xia et al., 2014). It will be with great interest to see the outcomes of these clinical trials.

1.6 Anti-tumour effects of aspirin

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that provide analgesic, antipyretic and anti-inflammatory effects, which include some well-known agents like aspirin, ibuprofen and naproxen. Mechanistically, these drugs are grouped together as their ability to inhibit the prostaglandin H (PGH) synthase--cyclooxygenase (COX) pathway thus blocking the COX-catalysed metabolism of arachidonic acid and down-regulating the level of prostanoids. Prostanoids are a group of biologically active signalling lipids including prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane A (TXA_2), which have profound effects in inflammatory response and vasoconstriction (Thun et al., 2012).

Among NSAIDs, aspirin differs from others in the way of inhibiting COX. COX has two identified isozymes, COX-1 and COX-2, which are derived from different genes. Both of them are expressed in most of tissues and are induced during inflammation response. COX-1 is the only COX isozyme expresses in platelet and plays essential role in platelet aggregation, while COX-2 is the primarily source of PGI_2 in vascular endothelium. Inhibition of COX by other NSAIDs is reversible, as they compete with arachidonic acid for binding to COX thus interfering the catalyse efficiency. In contrast, aspirin inhibits the activity of COX through acetylates serine 529 on COX-1 and serine 516 on COX-2 which makes this inhibition irreversible (Thun et al., 2012). Given the fact that platelets, unlike nucleated cells, can't synthesize COX isozymes,

this property gives aspirin special advantage in blocking platelets function thus preventing thrombosis or other related disorders (Thun et al., 2012).

1.6.1 Anti-tumour effects of aspirin

There is a considerable body of evidence supporting that long-term regular low-dose aspirin is effective in prevention and treatment of cancers, especially colorectal cancer (CRC) (Figure 1.8). The first epidemiological evidence about aspirin and other NSAIDs reduce cancer incidence in human beings arose about 28 years ago from a case-control study of 715 colorectal cancer cases compared to 727 age/sex-matched controls. In this study, which aimed to find link between CRC risks and various clinical conditions, they evidenced aspirin use significantly reduces colorectal cancer risks (Relative Risk [RR]=0.57, 95 % CI= 0.41-0.79, $p<0.001$), and there was a statistically borderline reduction in the incidences by taking NSAIDs (RR=0.77, 95 % CI= 0.60-1.01, p=0.06) (Kune et al., 1988). Subsequently, couples of placebo-controlled randomized trials (RCTs) in patients with previous CRC have implicated regular low-dose aspirin can effectively prevent occurrence of adenomas (RR=0.83, 95% CI = 0.72-0.96) and advanced-lesion occurrence (RR=0.72, 95% CI = 0.57-0.90) (Cole et al., 2009). CAPP2 study, a randomized trial aimed to investigate aspirin's effect on patients with high risk of hereditary colorectal cancer known as Lynch Syndrome, have indicated a relative high does (600mg daily) of aspirin taken for a mean of 25 months induce about 40% reduction of CRC incidence after a mean of 55·7 months follow up (Burn et al., 2011). More solid evidence of the aspirin benefits cancer incidences and mortality were provided by Rothwell and colleagues from a number of meta-analysis results of randomized trails (RCTs) designed originally to evaluate aspirin on vascular protection. Among these data, they demonstrated long-term allocation to daily low dose aspirin (up to 6 years) significantly reduced cancer death regarding all solid cancers types (Hazard Ratio [HR]=0·80, 95% CI= 0·72–0·88, $p<0·0001$) (Rothwell et al., 2011), and reduced CRC incidence (HR=0.76, 95% CI=0.60-0.96, p=0.02) and mortality (HR=0.65, 95% CI=0.48-0.88, p=0.005) after 20-year follow up period (Rothwell et al., 2010). Aspirin also induced about 40% reduction on the risks of cancer metastasis (HR=0.64, 95% CI=0.48-0.84, p=0.001) (Rothwell et al., 2012). Results from RCTs

are also supported by substantial observational studies. In 2006, a review of ~100 cohort and case–control studies up to 2005 demonstrated about 30% reduction on CRC risks with aspirin allocation (RR=0.71, 95% CI= 0.67–0.75) (Bosetti et al., 2006). Most importantly, a systematic comparison study suggests there is a strong agreement between results carried out by RCTs and case-control studies (Algra and Rothwell, 2012). Furthermore, more recent systematic reviews and meta-analyses on previously published studies also gave a more comprehensive review of all up to date RCTs and observational studies, and build up the notion that aspirin can substantially reduce cancer incidence and mortality, prevent cancer metastasis and spread (Chubak et al., 2015; Elwood et al., 2016). Besides colorectal cancer, several trials also suggested aspirin use might protect against other type of cancers, but these results remain provocative rather than decisive due to the limited number of trials and marginal effects (Thun et al., 2012).

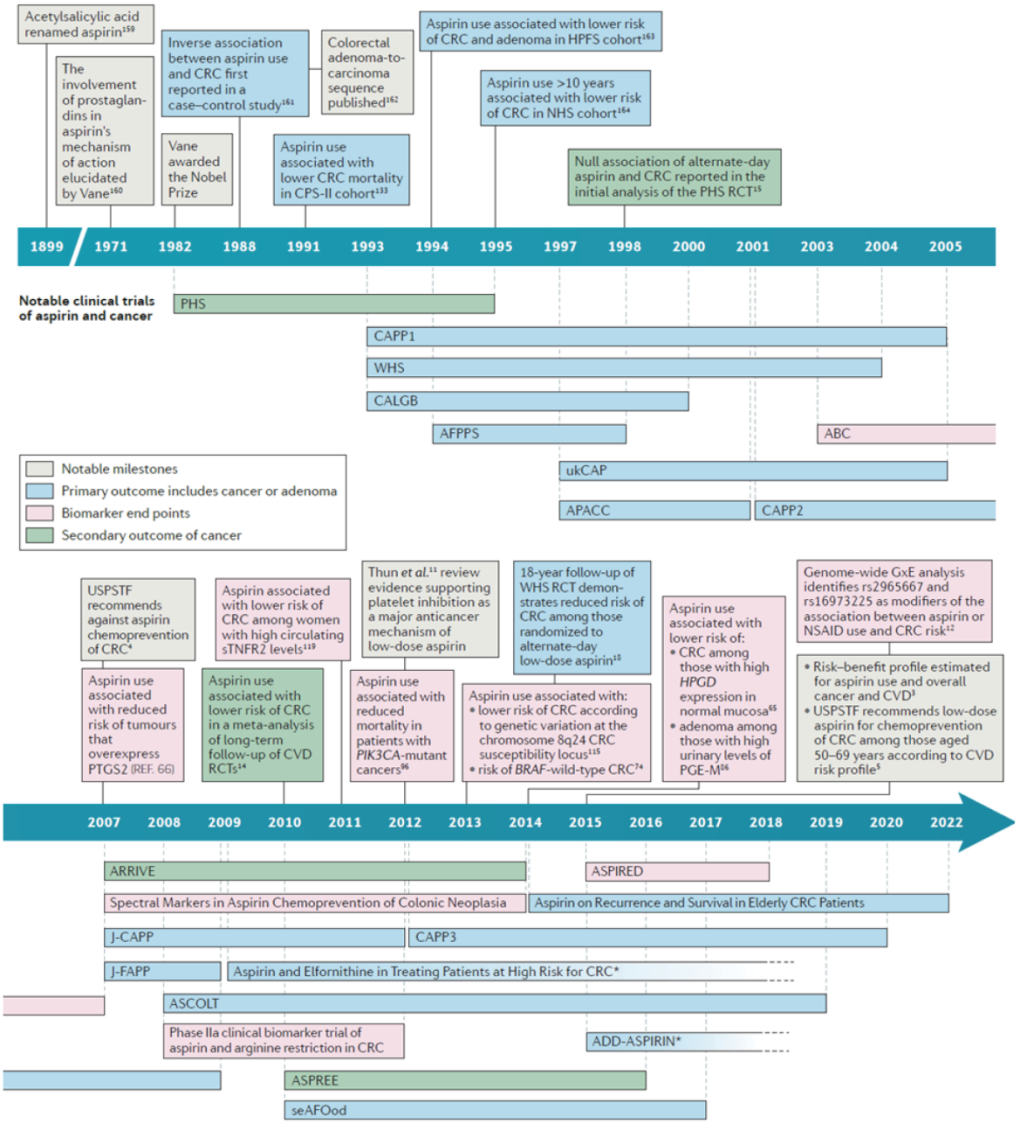


Figure 1.8 Milestones and notable studies of aspirin chemoprevention in human colorectal cancer (CRC). The diagram shows substantial epidemiological studies in past decades have compellingly demonstrated the anti-tumour effects of aspirin in CRC. Figure from Drew et.al, 2016.

Despite the substantial evidence convincingly show daily aspirin use reduces cancer risks, aspirin has not been widely considered by clinical guidelines as a chemopreventative agents. This is largely due to the following reasons:

1). Not all studies have shown the positive correlation between aspirin allocation and cancer prevention. Among them, two large randomized controlled trials from the Physicians' Health Study (PHS) and Women's Health Study (WHS) both failed to demonstrate aspirin's effect on cancer prevention. They found, following 5 years or

10 years alternate daily low dose aspirin treatment, there were no significant reduction in colorectal cancer incidence immediately at the end of randomized treatment or after a 12-year follow-up respectively (Cook et al., 2005; Sturmer et al., 1998).

2). Long-term use of aspirin brings major risks on intracranial haemorrhage, gastrointestinal bleeding and other kind of gastrointestinal complications (Thun et al., 2012). The most-important risk of long-term aspirin use is bleeding, mainly due to the inhibition of TXA_2 synthesis dysfunction platelets' activation and aggregation. In addition, result from the inhibition of PGE_2 synthesis, which has protective effects in the gastrointestinal tract, aspirin use increases the risks of gastrointestinal irritation. Many epidemiological studies have indicated the side effects associated with long-term use of aspirin thus provoking a question whether aspirin's cancer prevention benefits outweigh the harm associated with the long-term use of this agent (Thorat and Cuzick, 2015). Given this unclear balance, the U.S Preventative Service Task Force has advised against routine use of aspirin for primary chemoprevention of colorectal cancer in individuals at average risks (Chubak et al., 2015).

3). Optimised dose and treatment duration of aspirin on cancer prevention are still not available. Given the side effects of high-dose aspirin, daily low-dose aspirin use (75mg-300mg per day) was widely suggested and studied for the cancer prevention outcome. However, there are conflicts between studies. For example, there was a study shown dose more than 75mg cannot clearly increase aspirin's benefits (Rothwell et al., 2010), while another study evidenced a daily aspirin consumption at 600mg largely reduced the incidence of colorectal cancer in persons with Lynch Syndrome (Burn et al., 2011). For how long people need to take aspirin in order to efficiently prevent cancer is not conclusive yet, but it seems clear that aspirin has a delayed anti-tumorigenesis effect. As noted above, a ~30% reduction on colon cancer incidence, ~40% reduction on colon cancer mortality and ~40% reduction on the risk of metastasis was observed after a 20-year follow up study (Rothwell et al., 2010; Rothwell et al., 2012). And in particular, although the initial analysis of WHS data failed to demonstrate the positive role of aspirin on cancer prevention, a second analysis after 10-20 years did observed a significant reduction in mortality rate after

aspirin allocation (Rothwell et al., 2011). Moreover, studies also suggest a daily continuously uptake of aspirin benefits better than an alternate use (Thun et al., 2012).

4). The population groups that would benefit from aspirin most are unclear. Studies have suggested aspirin's cancer prevention benefit are unrelated to sex and smoking, but may maximally benefit older people at age >65 years (Rothwell et al., 2011). To further ensure a precise personalised assessment on the advantage and disadvantage of aspirin treatment, there are emerging studies on molecular and genetic level to investigate clues that would affect the outcome of aspirin (Coyle et al., 2016)(Drew et al., 2016). In particular, the status of *PIK3CA* gene has been linked to aspirin's clinical outcome. *PIK3CA* gene encodes PI3K, which is down regulated by aspirin and then inhibiting the activity of COX-2. A study in patients with diagnosed colorectal cancer has found, in patients carry mutant *PIK3CA,* aspirin treatment delivered a favourable outcome on cancer-specific survival (HR=0.18, 95% CI=0.06-0.61), while aspirin treatment was not associate with cancer-specific survival among patients with wide-type *PIK3CA* (HR=0.96, 95% CI=0.69-1.32) (Liao et al., 2012). The similar effect that genotypic differs clinical outcome of aspirin on colorectal cancer was also found on the genetic status of *BRAF*, gene encodes RAF kinases which promote COX-2 activity. As report by the same group, people with mutant *BRAF* were less sensitive to aspirin regarding colorectal cancer incidence (HR=1.03, 95% CI=0.76-1.38) compared to *BRAF* wide-type population (Nishihara et al., 2013). In addition, the T allele of the single nucleotide polymorphism (SNP) rs6983267 on chromosome 8q24 in individuals, along with the postivie nuclear expression of β-catenin, are also associated with positive effects of aspirin in lowering colorectal cancer risk (Odd ratio (OR)= 0.83; 95% CI = 0.74 to 0.94) (Nan et al., 2013). Furthermore, the overexpression of *PTGS2* gene, which encodes COX-2, was associated with favourable aspirin outcome in reducing colorectal cancer incidence (Positive PTGS2 (RR= 0.64; 95% CI=0.52-0.78) compared to negative *PTGS2* expression (RR= 0.96; 95% CI=0.73-1.26)) (Chan et al., 2007) (Figure 1.8).

5). Molecular mechanisms underlying the anti-tumorigenesis of aspirin are not completely understood. Given the promise of aspirin on cancer prevention and the disadvantages of it due to potential side effects, it is extremely necessary to deeply

understand the molecular mechanisms by how aspirin exert its anti-tumour effects in order to identify novel molecular targets of this agent and develop other drugs or combination strategy to build on the cancer prevention benefits.

1.6.2 Mechanism of anti-tumour action of aspirin

Although the molecular mechanisms of aspirin and other NSAIDs induced inhibition of platelets activity and inflammation reaction have been clearly established as described above, how they confer their anti-tumour action are still largely unknown. Currently, there are mainly two proposes to explain the chemoprevention action of these agents, dependent on whether inhibition on COX activity is required.

1.6.2.1 COX dependent pathways

Direct inhibition of COX activity has been pointed as the main mechanism by which aspirin and other NSAIDs use to prevent cancer development. This hypothesis has been supported by several epidemiologic and experimental studies.

Firstly, COX-2 level are increased in many solid tumour types, especially colorectal cancer. There was evidence that colorectal cancer patients with elevated COX-2 expression were associated with bigger tumours size, higher rate of metastasis and reduced survival. Therefore, COX-2 could be used a prognostic indicator of colorectal cancer (Soumaoro et al., 2004). Considering that colorectal cancer response best to low-dose of aspirin in cancer prevention, this study suggested the possibility that aspirin could target COX-2 expression for chemotherapeutic purpose. A robust support for this possibility comes from an epidemiologic study evidenced the cancer prevention outcome of aspirin use is strongly related to the COX-2 expression level. In this study, they applied immunohistochemistry assay in a cohort of colorectal cancer patients, and demonstrated aspirin use reduced colorectal cancer incidence in patients with overexpressed COX-2 (RR= 0.64; 95% CI=0.52-0.78) but not in patients with low expression or absence of COX-2 (RR= 0.96; 95% CI=0.73-1.26) (Chan et al., 2007). This has been supported by more recent case-control studies, which show the risk of colorectal cancer in response to aspirin are also associated with overexpression of other COX-2 pathway related metabolites, such as

hydroxyprostaglandin dehydrogenase 15-(NAD) (15-PGDH, HPGD) (Fink et al., 2014) and PGE-M (Bezawada et al., 2014).

Mechanistically, aspirin with low-dose and high-dose have been suggested to affect different steps of colon tumorigenesis through selectively inhibiting COX activity. In early stage of intestinal carcinogenesis, activated platelets-induced upregulation of COX-2 in stromal cells and intestinal epithelial cells promotes the transformation from normal mucosa to adenoma. Low dose aspirin may counteract the early events of neoplasia through a mechanism, by which inhibition of COX-1 in platelets and then subsequently suppress the induction of COX-2 in stromal cells and intestinal epithelial cells (Thun et al., 2012). This hypothesis is compatible with the findings from several RTC trials which prove low-dose aspirin can effectively prevent occurrence of adenomas (Cole et al., 2009), and using coxibs, a class of traditional NSAIDs selectively inhibit COX-2, generates the similar effects on prevention of adenomatous polyps (Arber et al., 2006). Experimental studies in mouse model of colorectal cancer further supports this hypothesis. For example, deletion of *COX-2* gene or applying COX-2 inhibitor dramatically decrease the number and size of the intestinal polyp in Apc delta716 knockout mice (Oshima et al., 1996); Homologous deletion either *COX-1* and *COX-2* gene reduced about 80% of the polyp formation in *Apc(Min/+)* mice (Chulada et al., 2000). Different from low-dose aspirin, aspirin used in anti-inflammatory high dose could inhibit COX-2 activity directly. Therefore, high-dose aspirin could target intestinal adenomas directly, which already have high levels of COX-2 expressed, thus inhibit the progression from adenomas to advanced lesions or metastasis (Dovizio et al., 2012).

However, given the fact that aspirin has a short half-life (15-20 minutes due to metabolism by liver and plasma esterases) and platelets in the portal circulation experience highest concentration of aspirin, the low-dose (75-300mg once daily) aspirin is considered only sufficient to irreversibly and selectively inhibits COX-1 in platelets and prevent thrombotic events, while just having minor effects on COX in nucleated cells (Thun et al., 2012). Besides, although high dose aspirin does achieve appropriate concentration to simultaneously inhibit COX-1 and COX-2, study has evidenced high doses don’t further reduce the cancer incidence and mortality

compared with low dose (Rothwell et al., 2010). Therefore, it is likely that other signalling pathways besides COX are effected by aspirin treatment and conduct the anti-tumour effects of the agent.

1.6.2.2 COX independent pathways

Despite the importance of COX activity in colon tumorigenesis and the accumulated evidence of inhibition of COX is responsible for aspirin and other NSAIDs' antineoplastic action, there are large amount of evidence have suggested a COX-independent way of this effect. The direct evidences of NSAIDs may act through COX-independent mechanism for their chemoprevention role come from a couple of studies, which demonstrated NSAIDs were effective in suppressing cell transformation and inducing cell apoptosis in COX null cells (Hanif et al., 1996; Zhang et al., 1999).

Through modulation on inflammatory and immune responses

Aspirin's anti-inflammatory properties and the well-established connection between chronic inflammation disease and colorectal tumorigenesis have strongly suggested a causal link between aspirin's chemopreventative effects and modulation on host immune response (Drew et al., 2016; Terzic et al., 2010). There are numbers of epidemiological studies have examined the association between CRC risk, cancer prevention outcome by aspirin and immune markers. For example, a case-control study in 32,826 women found the high plasma levels of the soluble tumour necrosis factor receptor 2 (sTNFR-2) was correlated to higher risk of CRC. The use of aspirin associated with lower risk of CRC among women with high baseline of sTNFR-2 (RR=0.39; 95% CI=0.18-0.86), but not among women with low baseline of sTNFR-2 (RR=0.86; 95% CI=0.41-1.79) (Chan et al., 2011). Similarly, the high levels of another inflammatory marker, macrophage inhibitory cytokine-1 (MIC-1 or GDF15), in plasma was linked to colorectal carcinogenesis as well. In an exploratory analysis of a cohort study, the same group also found high baseline of plasma MIC-1 was associated with reduced risk of COX-2-positive CRC upon regular use of aspirin (RR = 0.60; 95% CI=0.41-0.88) (Mehta et al., 2014).

A very recent Genome-Wide Association Study (GWAS) further implicated the role of aspirin in modulating immune response. In this genome-wide analysis of gene x environment interactions, two SNPs were identified showing genome-wide significant interactions with aspirin use. One of them, the SNP rs16973225 is located at chromosome 15q25.2 near the *IL-16* gene, which encodes IL‑16, a pleiotropic cytokine functions as a chemoattractant for T-cells and involves in T-cells activation. The case-only analysis in the study indicated regular use of aspirin was associated with a lower CRC risk among individuals with rs16973225-AA genotype (OR = 0.66; 95% CI=0.62–0.71; $P = 1.9 \times 10^{-30}$) but not among individuals with rs16973225-AC or CC genotypes (OR = 0.97; 95% CI 0.78–1.20) (Nan et al., 2015).

Taken these evidence into account, and together with a very early finding that aspirin induced antigen presentation proteins HLA-DR expression in colon cancer cells (Arvind et al., 1996), aspirin may modulate inflammatory or immune response and increase the immune surveillance for its cancer prevention purpose.

Through modulation on cellular signalling pathways

Studies up to date have indicated aspirin and other NSAIDs could alter numbers of cellular signalling pathways which are critical or associated with carcinogenesis process (Figure 1.9).

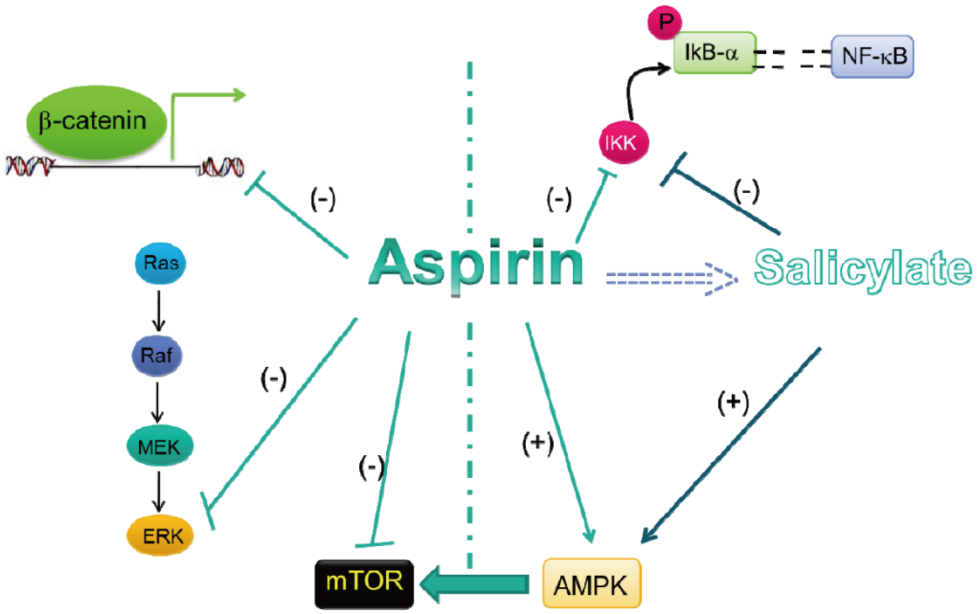


Figure 1.9 COX-independent anti-tumorigenic mechanisms of aspirin. The proposed schematic network summarises the studies demonstrated multiple signalling pathways, including Wnt/β-catenin, MAPK, mTOR, AMPK and NF-κB, are regulated by aspirin or salicylate and contribute to the anti-tumour effects of this agent. Figure adapted from Dovizio et.al, 2012.

Wnt/β-catenin

APC gene mutation has been well-known as a key factor drives initiation of colorectal cancer or adenomatous polyp formation. Familial adenomatous polyposis (FAP) is caused by germ-line *APC* mutations; by the age of 40 years old, the risk of colorectal cancer is almost 100% in *APC* gene mutation carriers (Markowitz and Bertagnolli, 2009). In most cases, a direct result of *APC* gene mutation is the dysregulation and activation of Wnt/β-catenin pathway. *APC* gene encodes APC protein, a tumour suppressor that negatively regulates Wnt/β-catenin signalling pathway. APC protein is a part of β-catenin 'destruction complex', which controls the cellular level of β-catenin by driving ubiquitination-dependent proteolysis of it. Loss of APC protein will cause abnormal high level of cytoplasmic β-catenin whose nuclear translocation activates the transcription of proliferative genes.

Given the importance of *APC* gene mutation and activation of Wnt/β-catenin pathway in colorectal cancer development, the interplay between aspirin and Wnt/β-catenin has been investigated to illuminate the anti-cancer mechanisms of aspirin

(Gala and Chan, 2015). Phosphorylation of β-catenin is crucial for its degradation. Bos CL et.al found millimolar level of aspirin *in vitro* inhibits the activity of Wnt pathway, evidenced by observations showing increased phosphorylation of serine 41/threonine 45 on β-catenin, enhanced ubiquitination-dependent degradation of β-catenin, reduced nuclear translocation of β-catenin and reduced gene expression of Wnt target genes. They also demonstrated aspirin-induced phosphorylation of β-catenin is a consequence of upstream inhibition on protein phosphatase 2A (PP2A) enzymatic activity by the reagent (Bos et al., 2006). In addition, aspirin can also target the downstream effectors of Wnt/β-catenin pathway, like Peroxisome proliferator–activated receptor-δ (PPAR-δ). PPAR-δ is highly expressed in colorectal cancer and this gene is a direct transcriptional target of Wnt/β-catenin signalling, therefore PPAR-δ is suppressed by functional APC. Activation of PPAR-δ associates with increased number and size of intestinal polyps in *Apc(Min/+)* mice (Gupta et al., 2004). Study found in *Apc(Min/+)* mice, traditional aspirin covalently attached with a NO releasing moiety (NO-ASA) efficiently dismisses intestinal tumorigenesis through its inhibitory effect on PPAR-δ expression (Ouyang et al., 2006). Considering that *APC* gene mutation and activation of Wnt/β-catenin pathway is crucial in the early stages of colorectal carcinogenesis, these studies would suggest the inhibitory effects of aspirin on adenoma formation (evidenced in the epidemiologic studies) are possibly in part through its regulation on Wnt/β-catenin pathway.

Notably, recent case-control studies have found the single nucleotide polymorphism (SNP) rs6983267 on chromosome 8q24, which is a CRC susceptibility locus, is associated with an advantage effect of aspirin in preventing colorectal cancer (OR= 0.83; 95% CI = 0.74 to 0.94) (Nan et al., 2013). The T allele of the rs6983267 impairs the binding of transcription factor 7 like-2 (TCF7L2) to β-catenin, thereby decreasing the expression of MYC oncogenes. Moreover, among the population that carries T allele of the rs6983267, the cancer preventative effects of aspirin are only limited to individuals with positive β-catenin expression. This study provides evidence for tailored aspirin treatment dependent on the status of rs6983267 and expression of β-catenin.

MAPK

Mitogen-activated protein kinase (MAPK) pathways are widely expressed signalling cascades involved in converting a diverse array of external stimuli (such as mitogens, stress factors and cytokines) to cellular responses that effect cell proliferation, differentiation, survival and apoptosis (Cargnello and Roux, 2011). MAPK pathways can be characterized into several subgroups, which conventionally comprise the extracellular signal-regulated kinases 1/2 (ERK1/2), Jun N-terminal kinases/stress activated protein kinases (JNKs/SAPKs), p38 kinase and ERK5. MAPK pathways share the same typically three-tiered activation process, as the MAP3Ks activated by phosphorylation or binding to a small GTP-binding protein upon the stimuli, activated MAP3Ks activate MAP2Ks by phosphorylation which sequentially stimulate MAPKs through dual phosphorylation on threonine and tyrosine residues (Cargnello and Roux, 2011).

Aberrantly activated MAPK pathways is an essential feature for many types of malignancies, and there are a growing number of MAPK specific inhibitors have been approved for cancer treatment. Genomic profiling also revealed that the mutations on MAPK pathway components, such as *BRAF* gene encoding RAF kinase which is an important regulator of MAPK pathway, are common in human tumours (Burotto et al., 2014). Interestingly, there was an analysis on a RCT of colorectal cancer patients, which shown patients with wide-type *BRAF* gene convert more reduction on cancer incidence after aspirin allocation (HR=0.73; 95% CI=0.64-0.83) than their *BRAF* mutant counterparts (HR=1.03, 95% CI=0.76-1.38) (Nishihara et al., 2013). This finding strongly suggested the possibility that aspirin could achieve chemotherapeutic purpose through modulating MAPK pathways. Indeed, there are numbers of *in vitro* studies have indicated that aspirin regulates MAPK pathways, for example modulating phosphorylation of ERK. In one study, aspirin induced phosphorylation of ERK was linked with the inhibition of melanogenesis in melanoma cells, and specific ERK phosphorylation inhibitor abrogated the anti-melanogenic effect of aspirin (Nishio et al., 2016).

In addition to ERK, aspirin also regulates p38 MARK activity. Evidenced by the hosting lab, aspirin activates the p38 MAPK pathway, which followed by

degradation of cyclin D1 degradation and NF-κB nucleolar translocation, is responsible for aspirin caused colorectal cancer apoptosis. To support this, chemical inhibitor of p38 MAPK has been shown abolished aspirin's effect on downstream NF-κB and cell apoptosis (Thoms et al., 2007b). Similarly, aspirin treatment in human vascular smooth muscle cells also increases the phosphorylation of p38 MAPK (Redondo et al., 2010). This set of data provides strong evidence that aspirin's modulation on MAPK pathways is a potential mechanism underlying the pro-apoptotic effects of this agent.

AMPK and mTOR

The 5'-adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways are two critical sensors involved in the fundamental regulation of cellular energy balance. They are regulated by various energetic factors, such as growth factors and amino acids, and accordingly converting these signals to control cellular metabolism and homeostasis. Defect on any of them will lead to metabolic disorders including obesity, type 2 diabetes and cancer. AMPK activity is mainly regulated by its conformational change upon binding to AMP or ADP that promotes phosphorylation and inhibits dephosphorylation at threonine 172. Therefore, AMPK can sense cellular energy change by monitoring ATP to AMP or ADP ratio (Inoki et al., 2012). There have been some direct evidence show aspirin activates AMPK. Hawley et.al demonstrated that salicylate, products from hydrolysis of aspirin *in vivo*, directly binds to and allosterically activates AMPK through inhibiting the dephosphorylation on threonine 172. They also indicated, this regulation is central for aspirin enhanced fat utilization and reduced circulating lipids in fat-feed mice (Hawley et al., 2012). This is in agreement with the finding that aspirin promotes hepatocellular lipid metabolism by upregulating the phosphorylation of AMPK (He et al., 2015).

mTOR pathway has close inter-connection between AMPK pathway in response to energetic factors. mTOR, as a serine/threonine kinase, is also a downstream targets of AMPK (Inoki et al., 2012). Beside, mTOR always couples with PI3K/Akt pathway in regulating ribosomal biogenesis and cancer development (Porta et al.,

2014). Interestingly, a recent study demonstrated mice carrying *PIK3CA*^{H1047R} gene mutation, which express constitutively active PI3K, develop human-like invasive adenocarcinomas in a Wnt-independent way. These PI3K mutation-initiated tumours are sensitive to PI3K/mTOR inhibition suggesting the dependence on the PI3K/mTOR pathway (Yueh et al., 2016). This study, together with a previous randomized controlled trial shown colorectal cancer patients with *PIK3CA* gene mutation are more sensitive to aspirin's pro-survival effects (Liao et al., 2012), strongly indicate PI3K/mTOR pathway is a potential target for aspirin. Indeed, study from Din et.al in colorectal cancer demonstrated aspirin both activates AMPK through increase AMP: ATP ratio and direct binding and reducing mTOR activity through inhibiting the mTOR downstream effectors--S6K1 and 4E-BP1. They also demonstrated this pathway is partially involved in aspirin-induced autophagy in colorectal cancer (Din et al., 2012). But not all research has supported this notion that activation of AMPK/mTOR contributes to aspirin's anti-tumour effects. Recently, Gao et.al found activation of AMPK-mTORC2-Akt-ERK1/2 axis and induction of MCL-1 expression compromises aspirin's pro-apoptotic effects, whereas combination treatment of aspirin and sorafenib (a kinase inhibitor block aspirin-induced MCL-1 upregulation) are more efficient in preventing hepatocellular carcinoma and colorectal adenomas(Gao et al., 2016).

NF-kappaB

As described in section 1.5 of this chapter, NF-κB signalling pathway is well known as its critical and pleiotropic role in various cell functions, especially inflammatory response and cancer development. Other than COX pathway, the role of NF-κB pathway in anti-tumour activity of aspirin has been most heavily investigated in the past 20 years.

There is a set of early studies have demonstrated that aspirin inhibits the activation of NF-κB pathway. The very first report described aspirin affects NF-κB transduction was in a study that investigated the anti-inflammatory nature of aspirin. Kopp et.al evidenced that *in vitro* anti-inflammatory doses of aspirin as well as sodium salicylate (up to 10mM) prevented the degradation of IκBα and nuclear translocation of NF-κB, and therefore inhibited the NF-κB dependent gene transcription in human

B cell and T cell lines under LPS- and phorbol 12-myristate 13-acetate (PMA) treatment (Kopp and Ghosh, 1994). A later study built on this concept by evidencing sodium salicylate inhibited TNFα-induced IκBα phosphorylation in endothelial cell line, resulted in the inactivated NF-κB pathway, reduced endothelial-leukocyte adhesion molecule expression and inhibition of transendothelial migration of neutrophils. They also noticed this inhibition was not due to the inhibition of COX pathway (Pierce et al., 1996). A subsequent study had gone deeper into the mechanism of this inhibition, established that aspirin and sodium salicylate specifically inhibited IKKβ activity through binding to IKKβ and competing with ATP for binding this kinase, thereby preventing IKKβ dependent phosphorylation of IκBα and activation of NF-κB pathway under TNFα treatment (Yin et al., 1998). Aspirin had also been reported inhibited degradation of IκBα through interfering with the proteasome function (Dikshit et al., 2006).

However, in contrast to these findings based on anti-inflammatory effect of aspirin, recent studies (including a body of studies in the hosting laboratory) about this agent's pro-apoptotic mechanism have suggested aspirin activates the NF-κB pathway. The hosting laboratory was the first to analyse aspirin and other NSAIDs' effects on NF-κB pathway and the association between these effects and cell death of colorectal cancer. In the absence of additional stimuli, they observed aspirin induced proteasomal-dependent degradation of IκBα and nuclear translocation of NF-κB in a time- and concentration-dependent manner. This activation was also evidenced associated with colorectal cancer cell apoptosis by applying a super-repressor form of IκBα which was resistant to stimuli-induced phosphorylation and degradation. They found cells constitutively expressed this super repressor IκBα were resistant to aspirin-induced NF-κB activation and most importantly apoptosis (Stark et al., 2001). Similar activation of NF-κB pathway was later observed by the same group *in vivo* in two mouse models of human colorectal cancer, in which they also demonstrated that even low-dose aspirin (40 mg/kg) could achieve the concentration of salicylate levels in xenografted tumours to activate NF-κB pathway and induce apoptosis of neoplastic epithelial cells (Stark et al., 2007).

Further studies from the host laboratory also elucidated the detailed molecular pathways underlying aspirin-induced NF-κB activation and revealed a novel mechanism explaining aspirin's pro-apoptotic action. They found, under the pro-apoptotic stimuli like aspirin, UV-C and serum starvation, NF-κB/RelA translocates to nucleus but is sequestered in nucleolus thus inhibiting NF-κB-driven transcriptional activity and inducing apoptosis. To the contrary, in response to stimuli like TNFα or TRAIL, RelA only accumulated in nucleoplasm and promoted NF-κB-driven transcriptional activity. They also identified the nucleolar localisation signal (NoLS) on RelA and deletion of which was evidenced inhibited aspirin induced RelA nucleolar sequestration and cell apoptosis (Stark and Dunlop, 2005). Subsequently, they examined and identified COMMD1, the component of the RelA ubiquitin ligase complex upregulated by aspirin, was a critical factor regulating ubiquitination and nucleolar translocation of RelA (O'Hara et al., 2014; Thoms et al., 2010). In addition, they draw the upstream pathway of this signal transduction network by revealing p38 MAPK-Cyclin D1/CDK4 axis and c-Src tyrosine kinase pathway act upstream and mediate aspirin-activated NF-κB (Brady et al., 2011; Thoms et al., 2007b). It is need to be addressed here that, although the mutation of tumour suppressor p53 has been widely studied and linked with colorectal tumorigenesis, the hosting laboratory has demonstrated aspirin-induced modulation on NF-κB and colorectal cancer cell apoptosis were irrespective to the p53 gene status (Din et al., 2004).

In addition to aspirin, the host laboratory also demonstrated that other NSAIDs (including sulindac, sulindac sulfone and indomethacin) stimulates NF-κB in colorectal cancers and suggested NF-κB as a common signalling target responsible for the chemoprevention effects of NSAIDs (Loveridge et al., 2008). This was supported by another study that demonstrated NSAID diclofenac promoted IκBα degradation and the nuclear levels of NF-κB. In this instance, they also postulated a crosstalk between NF-κB and Wnt/β-catenin pathway, as activation of NF-κB suppressed β-catenin driven transcription which is important in the early transformation of colorectal cancer (Cho et al., 2005).

Significance of aspirin's effect on NF-κB

Although it was contentious about the unique role of NF-κB in aspirin-mediated anti-inflammatory and anti-tumour response (as evidence shown aspirin and sodium salicylate non-specifically affected various cellular kinase activity (Frantz and O'Neill, 1995)), these body of researches did demonstrated NF-κB pathway was a molecular target of aspirin in a pharmacologically relative dose level. It is even promising about aspirin's potential modulation on NF-κB when we consider the pivotal role of NF-κB pathway in mediating inflammatory response and cancer development, and the significance that even a partial change of this pathway can have substantial effects on downstream cellular events.

In addition, what makes the investigations of aspirin's effect on NF-κB more interesting is, the host laboratory has indicated a cell-type specificity of aspirin-induced NF-κB activation (Stark et al., 2001) (Din et al., 2004). They observed that aspirin specifically stimulated NF-κB and induced apoptosis in all colorectal cancer cells rather than in normal intestinal mucosa (Stark et al., 2007) or in other non-gastrointestinal origins (Stark et al., 2001) (Din et al., 2004). They further underlined that aspirin stimulated NF-κB is a general effect in colorectal cancer, which is unrelated to COX-2 expression levels and gene mutation status of *APC*, *p53* or DNA mismatch repair (MMR) genes (Din et al., 2004). This finding, paralleled with the observations from abundant epidemiological studies that aspirin protects colorectal cancer to a greater degree than noncolonic cancers, strongly suggests activation of NF-κB as a central molecular rationale for the particular chemoprevention effect of aspirin against colorectal cancer.

In order to improve the benefits of aspirin in colorectal cancer prevention and develop tailored assessment on the advantage and disadvantage of aspirin treatment in individuals, recent case-control studies along with genomic analysis have identified several genetic markers that may confer the differential benefits from regular aspirin use (Figure 1.8). Notably, most of these identified markers have known associations with the NF-κB pathway. For example, TNFR2 and IL-16 are factors that are involved in immune response and regulating the NF-κB pathway. PI3K and MAPK pathways have well-establised inter-talks with the NF-κB pathway. It would be interesting to explore whether these genetic alterations are associated

with activation of the NF-κB pathway in these studies, and whether the activation of NF-κB is directly related to the outcome of aspirin treatment in colorectal cancer prevention.

Through modulation on cellular organelles

Intense researches on the molecular basis of aspirin and other NSAIDs' antineoplastic effects have clearly shown that multiple pathways are involved in these effects, but they display cell-type specificity and not all the pathways are shared by all the members of NSAIDs. Compared with signal transduction pathways, the formation and function of cellular organelles are more conserved across all tissues and cell-type thus could be potential targets of aspirin and other NSAIDs for delivering anti-tumour effects. In this part, I will mainly describe the evidence and the promising facts of mitochondria and nucleolus in the chemoprevention effects of aspirin.

Mitochondria

Mitochondria are the most recognized power plants in eukaryotic cells, and are now well established as a central controller of the mitochondrial apoptotic pathway (or intrinsic apoptotic pathway). In this intrinsic signalling pathway, the mitochondrial membrane permeability change will lead to the release of numbers of pro-apoptotic factors, such as cytochrome c, into cytosol where they activate the caspase cascade and promote apoptosis process (Suzuki et al., 2010). There is a large of body of evidence showing aspirin and other NSAIDs cause cell apoptosis through affecting mitochondrial functions.

Bellosillo et.al found aspirin induced a decrease in cell viability and cytotoxic through activation of caspase dependent pathway in B-cell chronic lymphocytic leukemia cells derived from patients (Bellosillo et al., 1998), and they later demonstrated this activation is a result of aspirin-induced mitochondrial membrane permeability change and release of cytochrome c (Pique et al., 2000). Meantime, in agreement with this finding, Zimmermann et.al evidenced aspirin induced cytosol to mitochondria translocation of BAX in HeLa cells, which then promoted cytochrome

c release from mitochondria and bind with pro-apoptotic factor Apaf-1 to activate downstream caspase cascade and induce cell apoptosis (Zimmermann et al., 2000). Notably, all of these studies have found caspase inhibitor, Z-VAD-FMK, blocked aspirin-induced cell apoptosis then strongly suggested the involvement of the caspase-dependent apoptosis pathway in the pro-apoptotic effect of this agent. Subsequently, Dikshit et.al demonstrated aspirin mediated changes in the mitochondrial membrane permeability, release of cytochrome c from mitochondria, and activation of caspase-9 and caspase-3 in mouse Neuro 2a cells and HeLa cells. Besides, an alternative aspirin-induced proteasomal malfunction mechanism was postulated in this study, as aspirin treatment decreased proteasome activity and increased the accumulation of ubiquitylated proteins in the cells, which paralleled with its effect on cell death (Dikshit et al., 2006). Study from the host laboratory shed light on a potential crosstalk between NF-κB pathway and mitochondrial apoptotic pathway in response to aspirin stress. They revealed a novel molecular path that nucleolar translocation of RelA promoted nucleolus to cytoplasm translocation of nucleolar protein nucleophosmin (NPM), which caused apoptosis by mediating the mitochondrial accumulation of BAX and was independent of NF-κB driven transcription (Khandelwal et al., 2011).

Mitochondria plays important role in regulating and maintaining cellular Ca^{2+} concentration. Imbalanced intracellular Ca^{2+} concentration has been associated with cell apoptosis (Suzuki et al., 2010). Núñez et.al proposed another mechanism facilitating aspirin mediated cell apoptosis through mitochondria. They found salicylic acid inhibited mitochondrial Ca^{2+} uptake by interrupting a Ca^{2+} influx pathway, Store-operated Ca^{2+} entry (SOCE), which correlated with impairment of cell growth and induction of cell death in Jurkat and human colon cancer cells (Nunez et al., 2006).

Nucleolus

The nucleolus has been comprehensively discussed in section 1.1 to 1.4 of this chapter. Considering its central role in sensing cellular stress and converting various signals into cellular function response, nucleoli could be an area of interest in the anti-tumour activity of aspirin. Indeed, work carried out in the past decade in the host

laboratory has clearly identified the nucleolus as a convergent point in aspirin-mediated NF-κB pathway modulation, with NF-κB/RelA sequestered in the nucleolus upon stimulation with aspirin, UV-C and serum starvation (Brady et al., 2011; Stark and Dunlop, 2005; Stark et al., 2007; Thoms et al., 2007b; Thoms et al., 2010).

In the course of these studies, the host laboratory surprisingly found that aspirin and other stresses that cause nucleolar accumulation of RelA, induce a distinct nucleolar stress. That is, increased nucleolar size decreased nucleolar number and repression of rDNA transcription (unpublished) (Figure 1.10 A-C). These modifications on nucleolar morphology are also observed in response to stresses like UV-C exposure and serum starvation. Furthermore, it was found that they preceded stimulation of the NF-κB pathway, as evidenced by degradation of IκBα and nuclear/nucleolar translocation of RelA (unpublished) (Figure 1.10 D and E).

These gave an exciting possibility that nucleolar stress may act upstream of NF-κB in response to aspirin. However, the molecular basis of aspirin's effect on nucleolus and whether aspirin-induced nucleolar stress is responsible for subsequent NF-κB activation and cell death are completely unknown. Although a diversity of cellular stresses converge on the NF-κB pathway, no common model has been found to mediate this activation. Interestingly, previous literature reviewed in the host lab revealed almost all the stresses that stimulate the NF-κB pathway also cause nucleolar disruption, although with different effects on the organelle (Table 3.1). Together with the preliminary data from the host laboratory, these data suggest that it would be really interesting to explore whether the nucleolus is a common sensor converting cellular stress to NF-κB signalling.

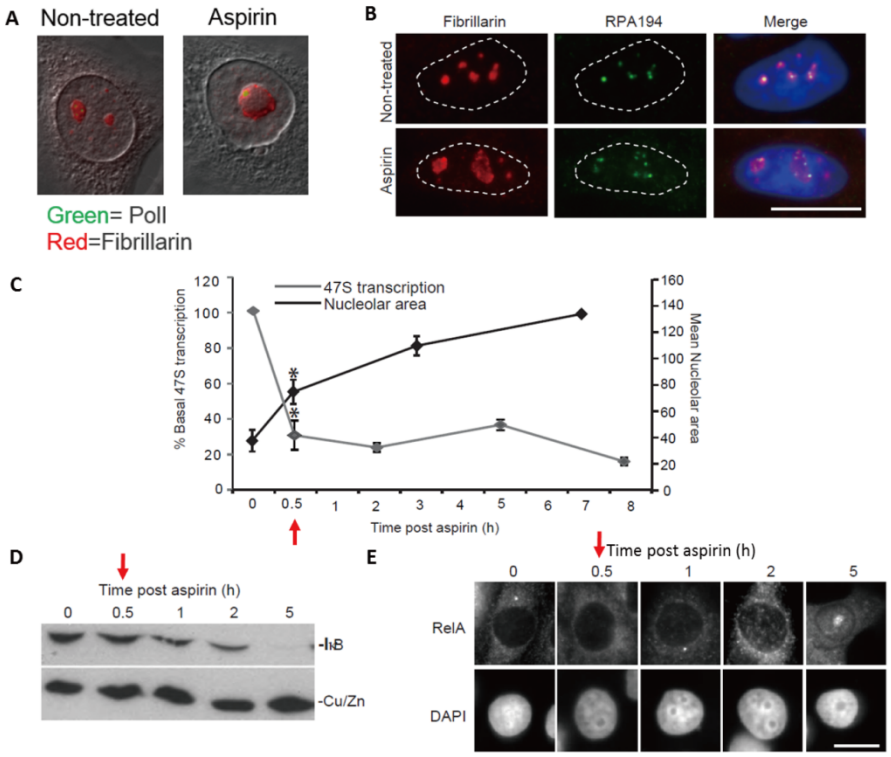


Figure 1.10 Aspirin effects on the nucleolus precedes activation of NF-κB. (A) shows enlarged nucleolus (red) in human CRC cell after aspirin treatment. (B) indicates enlargement of nucleolar size (red) and segregation of sub-nucleolar compartment (green) in response to aspirin. (C) Diagram shows aspirin induces enlargement of nucleolar size and inhibition of rDNA transcription in 30 minutes, which precedes degradation of IκBα (C) and nuclear translocation of RelA (D) under the same condition of aspirin treatment. Unpublished data. Experiments carried out by Jim Simpson in the lab.

Taken together, these preliminary data lead us to some very interesting scientific questions regarding the role of the nucleolus in the effects of stresses (such as the chemopreventative/therapeutic agent aspirin) on the NF-κB pathway and apoptosis. Given the crucial role of the nucleolus in multiple cell functions, further investigation into the effects of aspirin and other stress inducers on this organelle, and how these effects induce functional consequences, is desirable. This would not only provide further knowledge about the nature of nucleolus in regulating cell growth and cell death, but also may help us reveal additional molecular targets for targeting its function with novel anti-tumour agents.

1.7 Project Aims

This project used biomedical approches and mainly *in vitro* tumour cell line study to identify a novel nucleolar stress response pathway that culminates in the activation of NF-κB pathway and with a view to investigate the significance of this pathway in the anti-tumour effects of aspirin. The ultimate aim of this project is to identify potential molecular targets for novel anti-cancer agents and improving the chemoprevention efficiency of aspirin.

Aims of the project are:

1. To examine whether modulation of NF-κB pathway is a downstream consequence of nucleolar stress;
2. To elucidate the molecular basis that links stress stimuli, nucleolar response and stimulation of NF-κB pathway;
3. To test the significance of this nucleolar stress response pathway in pro-apoptotic effect of aspirin.

Chapter 2: Materials and Methods

2.1 Mammalian Cell culture and manipulation

2.1.1 Cell lines and maintenance

Different colorectal cancer cell lines were applied. SW480 cells, a Dukes' type B colorectal adenocarcinoma (ATCC® CCL-228™) and RKO cells (ATCC® CRL-2577™) were obtained from the American Type Culture Collection (ATCC). HRT18 parental cells and the counterparts IκBα mutant cells--IκBSR1 and SR28 clones (that are resistant to aspirin-induced nuclear and nucleolar translocation of RelA) were developed and characterised previously (Stark et al., 2001). The HCT116 wide-type and the p53 null derivative of HCT116 (HCT116 p53-/-) were gifts from Professor B Vogelstein (John Hopkins University School of Medicine, USA) and has previously been described (Din et al., 2004).

HeLa, Human Cervical cancer cells (ATCC® CCL-2™) and U2OS, Human Bone Osteosarcoma Epithelial Cells (ATCC® HTB-96™) are available from the American Type Culture Collection (ATCC).

Cells were grown in Leibovitz's L-15 medium (Gibco) (SW480 cells), RPMI1640 medium (Gibco) (HRT18 cells, HCT116 cells), Dulbecco's Modified Eagle Medium (DMEM medium) (Gibco) (RKO cells, HeLa cells, U2OS cells) respectively. Medium were supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin and streptomycin solution (10% FCS, p/s+ medium). Cells were cultured in T25 flask ($25cm^2$) or T75 flask ($75cm^2$) container (CellStar® Cell Culture Flasks) in 37°C 5% CO_2 incubator (Hera Cell 240, Heraeus, Germany).

Cells were passaged in the laminar flow cabinet (Heraeus, Germany). Cells were washed by 1 x phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM anhydrous; Na_2HPO_4, pH 7.4) followed by incubated with 1:1 tyrpsin:versene (T:V) at 37 °C for 10 minutes. Cells were maintented and passaged in a 1: 5- 1:20 dilution dependent on the further requirements.

2.1.2 Treatment procedure

Cells were counted by Coulter Counter (Z Series, Beckman Coulter, High Wycombe, UK). Since cell confluence in *in vitro* tissue culture system is a criterion that could potentially affect cell cycle progression and influence on Pol I transcription and differ the drug treatment outcome (Hannan et al., 2000), cells were seeded in an initially intensity of 3 x 10^4, 5 x 10^4 or 1 x 10^5 per cm^2 in 6 wells plate for the appropriate assays. 24 hours after seeding (cells with 50-80% cell confluent), cells were washed with 1 x PBS and changed medium to low serum medium (0.5% FCS medium supplemented with 1 % penicillin and streptomycin solution) prior to continuously exposure to specific drugs and indicated time duration. Immediately after treatment, cells were washed with 1 x PBS again at 4 °C to remove floating dead cells and debris, followed by harvesting or fixation for the appropriate assays.

2.1.3 Drugs

Aspirin was prepared as described in (Stark et al., 2001). Briefly, stock solution of aspirin (ASA; 0.5 M, pH 7.0; Sigma Aldrich, Gillingham, UK) was prepared by solubilising in distilled water (dH_2O), with 5 M NaOH used to adjust the pH appropriately.

The CDK4 inhibitors (2-bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione) (CDK4i, Calbiochem) was prepared by solubilising in dimethyl sulfoxide (DMSO, Sigma) to stock concentration 2mM. Stored in -20°C in darkness. Palbociclib (PD-0332991) (Selleckchem, Cat. No. S1116), another CDK4 inhibitor, was prepared by solubilising in DMSO to stock concentration 1mM. Stored in -20°C protected from light.

MG132: MG132 (Z-Leu-Leu-Leu-al) (Sigma Aldrich, C2211-5MG) was prepared by solubilising in DMSO to stock concentration 50mM. Stored in -20°C.

BMH-21 (12H-Benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide, N-[2(dimethylamino)ethyl]-12-oxo) (Kindly supplied by Prof. Marikki Laiho from Johns Hopkins University School of Medicine, USA)

CX-5461: CX5461 (Selleckchem, Cat.No.S2684) was prepared by solubilising in DMSO to 1mM stock concentration. Stored in -20°C.

Nutlin-3 (Kindly supplied by Prof. Kathryn Ball from Edinburgh Cancer Research Centre, UK)

Calyculin A (Cell Signaling Technology), Cycloxexamide (Sigma), Lactincystin (Calbiochem), Ceramide isoforms (Sigma), Bafilomycin (Sigma), Quinacrine (Sigma), TNFa (R&D Systems) and Actinomycin D (Sigma) were all prepared as per manufacturer's instructions.

2.1.4 RNA interfere

One day prior to siRNA transfection, cells were seeded in a 6-wells plate in an initial intensity of 3 x 10^4 per cm^2. Cells were then transfected with specific siRNA using Lipofectamine 2000 (Invitrogen) following the optimised manufacturer's instructions below. (Quantities indicated below are for transfection in a well of a 6-wells plate). For every transfection, 125 pM of siRNA designed against specific genes or scrambled siRNA were added to 312.5ul optimem (optimem reduced serum media (Invitrogen)). 6.25ul lipofectamine 2000 were gently mixed with 305.25ul optimem before leaving it static for 5 minutes in room temperature. The siRNA: optimem solution and lipofectamine 2000: optimem solution were then mixed 1:1 and incubated for 30 minutes in room temperature. In the meantime of incubation, cells were washed by 1 x PBS once and supplied with 1.875ml fresh medium without penicillin and streptomycin solution (10% FCS, p/s- medium). After 30 minutes' incubation, 625ul of lipofectamine 2000: siRNA mixture were added into each well and returned to 37 °C incubator. To enhance the gene knockdown efficiency, a second siRNA transfection was performed one day after following the same process as described above. Cells then grew in 37 °C incubator for another day before harvesting for subsequent experiment operation.

Table 2.1 siRNA species used for transfection

Name	Sequence (5'-3')	Manufacturer
TIF-IA	CUAUGUAGAUGGUAAGGUU	Sigma Aldrich
UBF	CCAAGAUUCUGUCCAAGAA	Eurofins MWG Operon
p14ARF	AAGACCAGGUCAUGAUGAUGG	Eurofins MWG Operon
CDK4 #1	AAGGCCCGUGAUCCCCACAGU	Eurofins MWG Operon
CDK4 #2	AAGCCGACCAGUUGGGCAAAA	Eurofins MWG Operon
Scrambled control	AGGUAGUGUAAUCGCCUUG	Eurofins MWG Operon

2.1.5 Plasmids transfection

One day prior to plasmids transfection, cells were seeded in a 6-wells plate in an initial intensity of 5 x 10^4 per cm^2. Cells were then transfected with indicated plasmids using Lipofectin (Invitrogen) following the optimised manufacturer's instructions below. (Quantities indicated are for transfection in a well of a 6-wells plate). For each transfection, 3ug of appropriate plasmids were mixed with 100ul optimem. 10ul of lipofectin were added to a separate 100ul optimem in the meantime. Plasmids: optimem and lipofectin: optimem solutions were incubated for 45 minutes in room temperature separately before mixing them 1:1 for another 15 minutes in room temperature. In the meantime, cells were washed by 1 x PBS twice and applied with 800ul optimem which was enough to cover whole well. Then 200ul plasmids: lipofectin mixture were added to each well and returned to 37 °C incubator. 6 hours after transfection, plasmids: lipofectin transfection mixture were removed and replaced by fresh 10%FCS p/s+ medium. Cells were then incubated in 37 °C for a further 24-72 hours before harvesting for subsequent experiment operation.

Table 2.2 Plasmids used for transfection

Plasmids	Constructed by /Manufacturer
pEGFP-C1	Commercially available from Clonetech
pEGFP-C1-hTIF-IA	Kindly provided by I. Grummt (German Cancer Research Centre)
pEGFP-C1- hTIF-IA-94-204aa	Dr. Pierre Morin
pEGFP-C1- hTIF-IA-94-302aa	Dr. Pierre Morin
pEGFP-C1- hTIF-IA-94-403aa	Dr. Pierre Morin
pEGFP-C1- hTIF-IA-Δ1-94aa	Dr. Pierre Morin
pEGFP-C1- hTIF-IA-S170A or S170E	Jingyu Chen
pEGFP-C1- hTIF-IA-S172A or S172E	Jingyu Chen
pEGFP-C1- hTIF-IA-S170/172A or S170/172E	Jingyu Chen
pEGFP-C1- hTIF-IA-S199A or S199E	Jingyu Chen
pEGFP-C1- hTIF-IA-T200A or T200E	Jingyu Chen
pEGFP-C1- hTIF-IA-S44A or S44D	Jingyu Chen
Flag-UBF wild type	Kindly provided by R. Voit (German Cancer Research Centre)
Flag-UBF S484A	Kindly provided by R. Voit (German Cancer Research Centre)
3 x κB ConA Luciferase Plamsid	Kindly provided by R.Hay (University of Dundee)
ΔκB ConA Luciferase Plasmid	Kindly provided by R.Hay (University of Dundee)
pCMV-β	Commercially available from Promega
Flag-p14ARF	Kindly provided by A. Lamond (University of Dundee)
pcDNA3-IκBα $^{s32/36}$	Kindly provided by R.Hay (University of Dundee)

2.2 Bacterial cell culture

2.2.1 Site-specific mutagenesis

The TIF-IA site mutant pEGFP fusion plasmids were generated based on the pEGFP-C1-hTIF-IA plasmid (kindly supplied by Professor. Ingrid Grummt from German Cancer Research Centre). Pairs of complimentary oligonucleotides containing the desired mutations were designed (list below). Primers were synthesised by Sigma-Aldrich.

QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used and the manufacturer's instructions were followed. Briefly, prepared the sample reaction (5ul of 10 x reaction buffer, 1ul of dNTP mix, 1ul (20uM) of oligonucleotide forward primer, 1ul (20uM) of oligonucleotide reverse primer, 1.25ul of *PfuUltra* HF DNA polymerase, 0.5ul (50 ng) of pEGFP-C1-hTIF-IA plasmids, 40.25ul ddH_2O to a final volume of 50ul) (pUC18 plasmids supplied in this kit was used as control plasmids of transformation), cycled the reaction using the parameters (95°C 30s, [95°C 30s, 55°C 1mins, 68°C 8mins][18 cycles], 68°C 10mins, 4°C 30mins) in PCR machine (PTC-225 Peltier Thermal Cycler, MJ Research). Then add 1ul of the Dpn I restriction enzyme directly to each reaction and incubate at 37°C for 1 hour to digest the nonmutated parental dsDNA plasmids. Transfer 10ul of DpnI digested reaction solution into 50ul thawed XL-1 blue supercompetent cells (supplied in this kit). Put the reaction on ice for 30 minutes before heat pulse the reaction at 42°C for 45 seconds then place for 2 minutes on ice. Add 0.5ml pre-warmed Mg^+ Lysogeny broth (LB) medium and incubate at 37°C for 1 hours with continuous shaking at 225rpm. Then gently span down bacteria with 3000rpm for 5 minutes, removed supernatant to 200ul and gently re-suspended bacteria pellets to spread on agar LB plates containing 50ng/ml kanamycin. Put the transformation plate at 37°C for more than 16 hours. Single colonies grew on the plate would be picked for further characterised (IGMM technical service for DNA sequencing) and application.

2.2.2 Plasmids transformation in DH5α E.coli

In order to produce sufficient plasmids stock for further application, chemically competent Subcloning Efficiency DH5α *Escherichia coli* (Invitrogen) were transformed by intaking exogenous plasmids following manufacturer's instructions. That is, 10ng of dsDNA plasmids were added into 50ul of commercial DH5α cells. Put cells and plasmids mixture on ice for 30 minutes before heat pulse the reaction at 42°C for 20 seconds. Cells were returned to ice for 2 minutes before adding 900ul pre-warmed LB medium to recover the cells in 37°C for 1 hours with continuous shaking at 225rpm. 100ul-200ul of the culture was then evenly spread onto agar LB plates containing appropriate antibiotics (100ng/ml ampicillin or 50ng/ml

kanamycin). To allow the single colony formation, leave the plate in 37°C incubator for more than 16 hours.

2.2.3 Growth of transformed E.coli

Single colony was picked by using a p10 tip and directly put into a tube containing 3ml LB medium with appropriate antibiotics (100ng/ml ampicillin or 50ng/ml kanamycin). The mini-culture was put into a 37°C incubator for 8 hours with continuous and vigorous shaking (250rpm). For expanded plasmids production, 500ul of the incubated mini-culture was inoculated into 200-500ml LB medium containing appropriate antibiotics (maxi-culture), which was subsequently incubated at 37°C overnight with continuous and vigorous shaking at 250rpm.

2.2.4 Preparation of plasmids (Maxi-prep)

Plasmids were prepared from overnight-incubated maxi-culture by using Plasmids Maxi Kit (Qiagen, 12163), the manufacturer's instructions were followed.

2.2.5 Storage of transformed E.coli and plasmids.

For long-time storage, transformed E.coli culture were 1:1 mixed with sterilized 50% glycerol solution, this mixture was then kept in -80°C. Plasmids were directly stored in -80°C condition for years.

2.3 Preparation and handling of nucleic acid

2.3.1 RNA extraction

Total RNA was extracted from cells using RNeasy mini kit (Qiagen, 74104) following the manufacturer's instructions. Briefly, Up to 1 x 10^7 cells were disrupted and homogenized in Buffer RLT. One volume of ethanol was added to the lysate, which creates conditions that promote selective binding of RNA to the RNeasy membrane in the spin column. All the samples were then transferred to the RNeasy Mini spin column. Multiple spins were then applied for washing away contaminants

(by buffer RW1 and RPE), and purified total RNA is eluted in RNase-free wate. Quantify and quality of RNA were measured on Nano-drop (Thermo Scientific).

2.3.2 Dnase I treatment and cDNA synthesis

To purify extracted RNA, Dnase I (RQ1 RNase-free DNase, Promega, M610A) was applied to eliminate genomic DNA as follow. Prepared the sample reaction (1ul Dnase I, 1ul 10 x 1ul 10 x Reaction/Dnase buffer (Promega, M198A), 1ug RNA in total volume of 7 ul H_2O), place in the reaction in water bath at 37°C for 45 minutes before adding 1ul RQ1 Dnase stop solution (Promega, M119A) and transferring all the volume above to a new PCR tube. Place it in PCR machine (PTC-225 Peltier Thermal Cycler, MJ Research) for another 10 minutes at 65°C. In order to synthesis complimentary DNA (cDNA), added the following 'reverse transcriptase mixture' directly into the reaction above (4ul 5 x M-MLV RT reaction buffer (Promega, M531A), 2ul Random primer (Promega, C118A), 2ul dNTP (10mM) (Roche, 11483188001), 1ul M-MLV Reverse Transcriptase (Promega, M170B), 1ul RNasin®Plus RNase Inhibitor (Promega, N261B)), gently and thoroughly mixed and returned the tube into PCR machine, set up cycling parameter as follow: 42°C 1 hour, 95°C 5 minutes, 4°C 30 minutes. Synthesized cDNA was stored at -20°C for further PCR measurement.

2.3.3 PCR

Normal PCR was performed following the standard PCR process. Prepared and well mixed reaction (5ul 10 x buffer, 1ul 10mM dNTP, 1.7ul 50mM $MgCl_2$, 1ul 20uM primer forward, 1ul 20uM primer reverse, 0.4ul Plantium Taq polymerase (Invitrogen), 1ul DNA template, 38.9ul ddH_2O) was placed in PCR machine (PTC-225 Peltier Thermal Cycler, MJ Research) to process the following cycles: 94°C 5 minutes, [94°C 1 minute, 58°C 1 minute, 72°C 30 seconds][25-32 cycles, dependent on the specific genes], 72°C 10 minutes, 4°C 1 hour.

2.3.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Taqman gene expression system (Thermo Fisher Scientific) or SYBR green based system were used to set up quantitative real-time polymerase chain reaction (qRT-PCR).

2.3.4.1 Quantitative real-time polymerase chain reaction (qRT-PCR) with Taqman gene expression system

Taqman gene expression system (Thermo Fisher Scientific) contains a pair of unlabelled traditional oligonucleotide primers targeting a protein-coding transcripts and an oligonucleotide probe, which binds to a specific region that lies within the amplicon, with a fluorophore (FAM™ or VIC® dye) covalently attached to the 5' end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. The fluorescence emitted by the fluorophore on the probe is quenched by the quencher based on FRET (Fluorescence Resonance Energy Transfer) principal when they are in proximity. During amplification, as the primers being extended, the 5′–3′ exonuclease activity of Taq polymerase degrades the probe and removes the fluorophore reporter from the probe. The break of the proximity between reporter and quencher allows the fluorescence of fluorophore become detectable. Therefore, the intensity of detected fluorescence is proportional to the amount of DNA template amplifications.

qRT-PCR was set up in 384 wells plate (Thermo Scientific). Each qRT-PCR reaction contains the following components: 5ul Taqman Gene Expression Master Mix (Applied Biosystems, 4369016), 0.5ul primers + probe, 2.5ul Rnase/Dnase-free ddH_2O (Gibco, 1097-035), 2ul cDNA template. Plate was then placed in the lightcycler HT7900 (Life Technologies) and cycled the reaction using the parameters (95°C 10 minutes, [95°C 10 seconds, 60°C 30 seconds][45cycles]).

Table 2.3 Primers and probes used in Taqman gene expression assay

Gene Name	Primer sequence (5'-3')	Probe sequence	Manufacturer Info.
47s pre-rRNA	Forward-CGGGTCCGGGTCTCTGA Reverse-GCCCGCAGCGGAGA	FAM-CTTCCCCGCCGCCCC-NFQ	Life Technologies
p14ARF	N/A	N/A	Life Technologies (Order No. HS99999189_m1)
GAPDH	N/A	N/A	Life Technologies (Cat# 4352934E)
IκBα	N/A	N/A	Life Technologies (Order No. Hs00355671_g1)
Bcl-xL	N/A	N/A	Life Technologies (Order No. Hs00236329_m1)
Bax	N/A	N/A	Life Technologies (Order No. Hs00180269_m1)
XIAP	N/A	N/A	Life Technologies (Order No. Hs00745222_s1)
FAS	N/A	N/A	Life Technologies (Order No. Hs00236330_m1)

2.3.4.2 Quantitative real-time polymerase chain reaction (qRT-PCR) with SYBR green.

SYBR green is a fluorescent DNA binding dye which preferentially binds double–stranded DNA by intercalating between base pairs, and fluoresces only when bound to DNA. Therefore, the amount of the SYBR green emitted fluorescence and the amplified double-stranded DNA in the sample is in a direct proportion.

qRT-PCR was set up in 384 wells plate with each reaction contains the following components: 5ul 2 x SYBR Green Mix (Qiagen), 0.175ul 20uM forward primer (Sigma), 0.175ul 20uM reverse primer (Sigma), 3.65ul Rnase/Dnase-free ddH_2O, 1ul (50ng) cDNA template. Plate was then placed in in the lightcycler HT7900 (Life

Technologies) and cycled the reaction using the parameters (95°C 10 minutes, [95°C 10 seconds, 60°C 30 seconds][45cycles]).

Primers used for SYBR green based qRT-PCR (IL-6, IL-8, IL-11, IL-32) were kindly supplied by Dr. Simon Wilkinson lab (Edinburgh Cancer Research Centre, UK).

2.3.4.3 Data analysis

Data analysis were based on Ct value. ddCt algorithm was applied for quantifying relative changes in gene expression compared to control samples (siControl or non-treated control) (Chang et al., 2009). Absolute quantification using the standard curve method was also utilised in some experiments, for example examining the difference of specific gene expression levels in individual cell types (Pfaffl, 2001). Mean values (added up all values and then divided by the number of repeats) are represented in graphs where there are more than one values. Standard deviations (SD) are also calculated for samples where there are more than one values.

2.3.5 Storage of RNA and cDNA

Samples of RNA were stored in -80°C, cDNA were stored in -20°C.

2.4 Preparation and handling of protein

2.4.1 Soluble protein extraction from whole cells

In order to prepare protein samples for further analysis, whole cell lysis buffer (10 x Whole Cell Lysis Buffer, Cell Signalling Technology) was applied following the manufacturer's instructions. 1 x Whole Cell Lysis Buffer was prepared in advance by mixing the following components: 100ul 10 x Whole Cell Lysis Buffer, 10ul 100 mg/ml Pefabloc SC (Roche, 11429876001), 40ul 25 x Complete Protease Inhibitors Cocktail (Roche, 11873580001), 1ul 1mM Pepstatin A, 2ul 500mM Na_3VO_4, 10ul 100mM NaF, 1ml ddH_2O. Add 100ul 10 x phosSTOP (Roche) if phosphorylated proteins need to be analysed. Cells, which were treated as indicated in a well of 6-wells plate, were washed by 2ml pre-cold 1 x PBS twice before removing the cell culture medium. Then add another 1ml pre-cold 1 x PBS to each well and cells detached from well using disposable cell scraper (Greiner Bio-One, 541070) and

decanted into a 15 ml Falcon tube. Pelleting cells by centrifuging at 500G for 10 minutes in 4°C. 40-80ul 1 x Whole Cell Lysis Buffer was applied to re-suspend cell pellets and transferred into a new micro-centrifuge tube. Incubate the tube on ice for 30 minutes with vigorous vortex every 10 minutes. Then centrifuge at 12000 rpm for 6 minutes to pellet insoluble fragments, collected supernatant as the soluble protein extraction stock. This soluble protein extraction from whole cells was stored in -20°C for further analysis.

2.4.2 Cytoplasmic extraction

For specified experiments, in which cytoplasmic proteins and nuclear proteins need to be extracted separated, the following procedure was followed. Cells with specified treatment in 6-wells plate (with 100% confluent) were washed by pre-cold 1 x PBS twice before removing cell culture medium. Cells were then detached by using disposable cell scraper in 1ml 1 x PBS and decanted into a micro-centrifuge tube. Spin down the cell pellets for 10 min at 500G in 4°C. Then discarded supernatant and re-suspended each pellet from a well in 30 ul Cytoplasmic Extraction Buffer with NP40 (CEB buffer + NP40) (0.1% NP40, 10mM HEPES PH 7.4, 60mM KCl, 1mM DTT, 1x Complete Protease Inhibitors Cocktail (Roche), 1mg/ml Pefabloc SC (Roche), 1uM Pepstatin A, 1mM EDTA, add ddH_2O to final volume 1ml)(Add 1 x phosSTOP if phosphorylated proteins need to be analysed). Vigorous vortex the tube for 5 seconds before leaving on ice for 3 minutes. Tubes were then centrifuged at 1200 G for 5 minutes in 4°C. The clear supernatant here contains the crude cytosolic and loosely-associated membrane proteins and the pellet here contains nuclei and remaining cell debris. Decanted the supernatant to a new micro-centrifuge tube and re-spin it for 20 min at 7000G to pellet and separate any contaminating nuclei, collected supernatant here as cytoplasmic extract stock (CYT) and stored in -80°C. To dissolve the nuclear proteins, washed the pellet once in CEB buffer without NP40 and re-collected the pellets by spinning at 1200G for 5 minutes in 4°C. Then re-suspended the pellet in 50 ul Nuclear Resuspension Buffer (NRB) (20mM Tris-HCl PH8, 1mM $MgCl_2$, 600mM KCl, 1mM DTT, 25% Glycerol, 1x Complete Protease Inhibitors Cocktail (Roche), 1mg/ml Pefabloc SC (Roche), 1uM Pepstatin A, 1mM EDTA, add ddH_2O to final volume 1ml)(Add 1 x phosSTOP if phosphorylated

proteins need to be analysed). Vigorous vortex the tube for 5 seconds before alternately freezing and thawing the tube 3 times in dry ice or -80°C freezer. To collect the nuclear extract (NUC), centrifuge the tube for 20 min at 7000G in 4°C. Nuclear extracts were stored in -80°C.

In order to prevent gelling of NUC fractions when SDS-PAGE samples were prepared, added 1 ul RQ1 DNase (1unit) (Promega) per sample when the samples were in preparation for loading.

2.4.3 Insoluble proteins

Some of the proteins in cells were becoming aggresome and not soluble with the method mentioned in 2.4.1. The protocol here purifing aggresomes to get detergent insoluble proteins is adapted from an established method (Song et al., 2008). Cells were cultured in 10mm cell culture dishes and treated as specified as mentioned in 2.1.2. Removed the medium and washed cells after treatment with 10ml chilled PBS (+200mM iodoacetamide) twice. Then, added 1ml Disruption Buffer (DB buffer: 20mM Tris/HCl, PH 7.5, 2mM EDTA, 150mM NaCl, 1.2% Sodium deoxycholate, 1.2% Triton X-100, 200mM iodoacetamide (184.96g/mol), 1 x Protease Inhibitors Cocktail (Roche), 1mg/ml Pefabloc SC (Roche), 1uM Pepstatin A, 1mM Na_3VO_4, 1mM NaF) into dish, detached cells and transfer them into a 1.5ml microcentrifuge tube. Sonicated the sample in a sonicater with parameter setting: high power, 7*7s pulses with 15s break between) in chilled metal block. Centrifuged at 13000rpm for 15 minutes in 4°C. Pellets were then washed twice by 200ul DB buffer before centrifuged again used the same centrifuge condition. Removed supernatant and followed by suspending pellets in 25ul SDS loading buffer (1ml glycerol, 500ul b-Mercaptoethanol, 2ml 10% SDS, 1.25ml 1M Tris-HCl PH6.8, 3ml 0.05% Bromophenol Blue). Incubated sample in 95°C for 5mins and forwarded to western blot analysis.

2.4.4 Protein quantification by Bradford assay

Prepare 1 x Bradford Assay Reagent by diluting 5x stock (Bio-Rad, Hemel Hempstead, UK) with ddH_2O. Diluted reagent was filtered using Stericup/Steritop device (Millipore, Consett, UK) prior to use. Bovine serum albumin (BSA) was

made up to 1mg/ml in ddH_2O in advance for generating a standard curve. This assay was performed in a flat bottom 96 wells plate. A layout of 1mg/ml BSA loading for standard curve is shown below:

	1	**2**	**3**	**4**	**5**	**6etc**
A	0	0	0			
B	0	0.2ul	0.2ul			
C	0	0.5ul	0.5ul			
D	0	0.8ul	0.8ul			
E	0	1.0ul	1.0ul			
F	0	1.5ul	1.5ul			
G	0	2.0ul	2.0ul			
H	0	0	0			

Samples (1ul) were placed anywhere else on the plate in triplicate. Then added 200 µl 1 x Bradford Assay Reagent into all wells. Place the plate on a plate shaker for several seconds before incubating in room temperature for 10 minutes. Place the plate onto a Labsystems Multiskan MS Plate Reader, absorbance at 620 nm was measured and the concentration of protein samples (ug/ul) were calculated and output by extrapolation from the standard curve.

2.4.5 SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis)

Mini-PROTEAN ® Electrophoresis System (Bio-rad) was used for separation of protein samples by denaturing SDS-PAGE. Freshly-made gels were poured into a glass-plate-formed groove in advance which contain an upper layer of stacking gel on the top of a lower layer of resolving gel (see below for the formation of stacking gel and resolving gel). Combs (1.0mm or 1.5mm with 10 or 15 wells could be chosen

for appropriate experiments) (Bio-rad) were used to build separated lanes for loading samples.

Sample preparation and electrophoresis

Protein samples were prepared and denatured by adding 3ul SDS loading buffer (1ml glycerol, 500ul b-Mercaptoethanol, 2ml 10% SDS, 1.25ml 1M Tris-HCl PH6.8, 3ml 0.05% Bromophenol Blue) into 22ul (5-30ug) cell lysate. Samples were incubated at 95-100°C for 5 minutes in a heating block before loading. Each well of 1.0mm 10-wells comb could be loaded maximum 25ul of samples. PageRuler Plus Prestained protein ladder (Thermo Scientific) was loaded in a lane beside samples to identify proteins of interest by molecular weight. Electrophoresis carried out in a Mini-PROTEAN Tetra Cell filled with 1 x Running buffer (10 x Running buffer: 30g Tris-base, 144g Glycine, 100ml 10% SDS, add ddH_2O to 1L total volume) at 100V-150V for 1.5-2 hours until the blue bromophenol dye reached the bottom of the glass plates.

Formation of Resolving gel (volume specified are for 2 gels)

GEL %	8.0	10.0	12.0	12.5
ddH_2O (ml)	5.406	4.806	4.206	4.056
4 x Resolving Buffer (ml)	4.000	4.000	4.000	4.000
40% acrylamide (ml)	2.400	3.000	3.600	3.750
10% APS (ml)	0.182	0.182	0.182	0.182
TEMED (ml)	0.012	0.012	0.012	0.012
TOTAL (ml)	12	12	12	12

1. 4x resolving buffer: 18.17g Tris-base, 4ml 10% SDS, add ddH_2O to 100ml total volume.
2. 40% (w/v) acrylamide: Acrylamide Bis-Acrylamide stock solution (Severn Biotech Ltd. Cat No. 20-2400-05)

3. 10% APS (Ammonium Persulfate): 1g ammonium persulfate in 10ml ddH_2O
4. TEMED (N,N,N',N'-Tetramethylethylenediamine) (Sigma-Aldrich, T9281)

Formation of Stacking gel (volume specified are for 2 gels)

ddH_2O (ml)	3.243
4 x Stacking Buffer (ml)	1.332
40% acrylamide (ml)	0.654
10% APS (µl)	84
TEMED (µl)	5

1. 4x stacking buffer: 6.07g Tris-base, 4ml 10% SDS, add ddH_2O to 100ml total volume.
2. TEMED (N,N,N',N'-Tetramethylethylenediamine) (Sigma-Aldrich, T9281)

2.4.6 Western blot analysis

Western blot was performed by a standard process. Briefly, after SDS-PAGE, proteins were transferred onto PVDF transfer membrane (Thermo Scientific, Prod 88518) by using Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-rad) filled up with 1 x wet transfer buffer (100ml 10 x Wet Transfer (30g Tris-base, 144g Glycine in 1L ddH_2O) + 100ml Methanol + 800ml ddH_2O) at 100V for 60-90 minutes. Transferred membrane was blocked in a solution with 5% no-fat dried milk in PBST (0.1 % TWEEN® 20 (Sigma) in PBS) at 4°C overnight. Then incubate the blocked membrane with primary antibody (Details of antibodies been used and dilution rate applied are summarized below) with appropriate dilution in 5% no-fat dried milk in PBST for 1 hour at room temperature. Washed away non-specific binding for 30 minutes (3 x 10 minutes) by using PBST. Membrane was subsequently incubated with secondary antibody conjugated to horseradish peroxidase diluted in 5% no-fat dried milk in PBST for 45 minutes at room temperature. 3 x 10 minutes PBST washing were then followed before using a 1:1 mixture of ECL reagents (Santa Cruz Biotechnology) to detect bounded antibody. Western blot bands on the membrane

were output by either using Fuji medical X-RAY films (Super Rx-N, Cat. No. 47410 19289), which was exposed and developed by a SRX-101A X-ray film processor (Konica Minolta), or directly detected and pictured by camera of Image Quant LAS4000 (GE Healthcare Life Sciences) (Method: Chemiluminescence; Digitization: Epi-illumination).

Table 2.4 Antibodies used for western blot

Antibody	Host Species	Dilution[1]	Incubation time	Manufacturer Info.
Anti-TIF-IA	Rabbit	1:2000	1 hour at RT[2]	BioAssayTech, B8433
Anit- Rrn3	Mouse	1:500	1 hour at RT	Santa Cruz Biotechnology, sc-390464
Anti- GFP	Rabbit	1:1000	1 hour at RT	Santa Cruz Biotechnology, sc-8433
Anti- UBF	Mouse	1:500	1 hour at RT	Santa Cruz Biotechnology, sc-13125
Anti-p53	Mouse	1:2000	1 hour at RT	Oncogene, OP43
Anti-IκBa	Sheep	1:5000	1 hour at RT	Gift from Prof. Ron Hay (University of Dundee)
Anti-Cyclin	Rabbit	1:2000	1 hour at RT	Thermo Scientific, OM-

D1				9104-S1
Anti-RPA194	Mouse	1:500	1 hour at RT	Santa Cruz Biotechnology, sc-48385
Anti- CDK4	Mouse	1:250	1 hour at RT	BD, 559677
Anti- p14ARF	Mouse	1:200	Overnight at 4°C	Thermo Scientific, MA5-14260
Anti-Capsesin	Mouse	1:1000	Overnight at 4°C	Gift from Prof Kathryn Ball (ECRC)
Anti- b-actin	Mouse	1:5000	1 hour at RT	Santa Cruz Biotechnology,
Anti- Mouse IgG (HRP conjugated)	Donkey	1:1000	45 minutes at RT	GE Healthcare UK Limited, NA9310V
Anti- Rabbit IgG (HRP conjugated)	Donkey	1:2000	45 minutes at RT	Promega, W401B
Anti- Sheep IgG (HRP conjugated)	Donkey	1:1000	45 minutes at RT	Abcam, ab6900

2.4.7 Co-Immunoprecipitation (Co-IP)

In order to examine protein-protein interaction, Co-immunoprecipitation was performed as followed. Magnetic beads (Dynabeads®, Novex) were used in this

protocol. Colorectal cancer cells were seeded into T75 (75cm^2) flask and followed by specified treatment. Cells were normally >90% confluent in a T75 flask when Co-immunoprecipitation carried out. Removed medium and washed cells with pre-cold 1 x PBS before adding 1.5ml NP40 cell lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Nonidet P40 (NP40), 1 x Protease Inhibitors Cocktail (Roche), 1 x phosSTOP (Roche), 1mg/ml Pefabloc SC (Roche), 1uM Pepstatin A, 1mM Na_3VO_4, 1mM NaF) directly into the flask. Incubated cells with NP40 cell lysis buffer on ice for 5 minutes followed by detaching cells using disposable cell scraper and decanting to a 1.5ml microcentrifuge tube. Incubated tube on a roller (Scientific Laboratory Supplies) in 4°C for 30 minutes to gently and thoroughly solve the cells. Harvested the supernatant containing the soluble proteins by centrifuging at 13000rpm for 5 minutes in 4°C. The supernatant was then incubated with 10ul Dynabeads® on a roller in 4°C for 1 hour to pre-clear the factors that can non-specifically band to the beads. Placed the tube on a magnet rack (DynaMag™-2 Magnet, Thermo Fisher Scientific, 12321D) to separate the beads from the solution and kept the supernatant. Performed Bradford Assay as stated in 2.4.4 and diluted protein concentration to 1 or 2mg/ml with NP40 cell lysis buffer. Kept 25ul samples here as Input control. Added 2ug specified antibody into 500ul samples solution (0.5 or 1mg proteins), returned sample tube to the roller in 4°C for incubating overnight. Then added 50ul Dynabeads® into the sample tube which contained antigen-antibody complex, return tube to the roller in 4°C to incubate for 4 hours to allow Dynabeads® bind to antigen-antibody complex. Placed the tube on the magnet rack and removed supernatant before washing the Dynabeads®-antibody-antigen complex three times using pre-cold 1 x PBS, separated on the magnet rack between each wash, removed supernatant and resuspended by gentle pipetting. To elute the target antigen, 30ul elution buffer (20ul 1 x PBS + 10ul SDS loading buffer (1ml glycerol, 500ul b-Mercaptoethanol, 2ml 10% SDS, 1.25ml 1M Tris-HCl PH6.8, 3ml 0.05% Bromophenol Blue) was added and thoroughly mixed by vortex followed by incubation in 90°C for 8 minutes. Put tube on the magnetic rack again to collect supernatant for further SDS-PAGE or western blot analysis.

Table 2.5 Antibodies used for Co-IP

Antibody	Host Species	Manufacturer Info.
Anti-Rrn3 (TIF-IA)	Mouse	Santa Cruz Biotechnology, sc-390464
normal mouse IgG	Mouse	Santa Cruz Biotechnology, sc-2025

2.5 Imaging

2.5.1 Immunocytochemistry

Cells were grown in wells of 6-wells plate with sterilised coverslips placed in and were treated as specified. Briefly washed cells with pre-cold 1 x PBS and added 3 ml Methanol: Acetone (1:1) to fix cells in -20°C for 30 minutes. Then removed methanol-acetone and gently washed with 1 x PBS for 3 times for 30 minutes on a shaker at room temperature before adding 2 ml 10% donkey serum (Sigma; diluted in PBS) to each well and incubated for 30 minutes at room temperature to block non-specific binding. Cells were then incubated with 200ul of the primary antibody with 1:200 diluted in 10 % donkey serum in room temperature for 1 hour. Post primary antibody incubation, cells were washed by PBST (0.1 % Tween-20 in 1 x PBS) 3 times for 30 minutes. Incubated cells for 30 minutes at room temperature in darkness then in the presence of 200ul secondary antibody 1:200 diluted in 1.5 % donkey serum, which followed by another 3 x 10 minutes washes in the dark by PBST. Coverslips were then mounted in Vectashield containing 1ug/ml DAPI (Vector Laboratories, Inc., H-1200), which stain DNA, by placing cells facing down onto on the slides after carefully cleaning the back of the coverslip. Left 15 minutes to set and forward to microscope analysis directly or stored in darkness at 4°C for further analysis.

Table 2.6 Antibodies used for immunocytochemistry

Antibody	Host Species	Dilution rate	Manufacturer Info.
Anti-TIF-IA	Rabbit	1:200	BioAssayTech, B8433
Anti-RelA (C20)	Rabbit	1:200	Santa Cruz, SC-372
Anti-Fibrillarin	Mouse	1:200	Cytoskeleton, AFB01

2.5.2 Fluorescence microscope

Images were captured using a Coolsnap HQ CCD camera (Photometrics Ltd, Tuscon, AZ, USA) Zeiss Axioplan II fluorescent microscope, 63 × Plan Neofluor objective, a 100 W Hg source (Carl Zeiss, Welwyn Garden City, UK) and Chroma 83 000 triple band pass filter set (Chroma Technology, Bellows Falls, UT, USA). Image capture was performed using scripts written for IPLab Spectrum 3.6 or iVision 3.6 in house. For each experiment, a constant exposure time was used and at least 10 distinct fields were observed and recorded for each sample.

2.6 Others

2.6.1 NF-κB and gene reporter assay

NF-κB reporter assay and gene (IκBα, Bcl-xl) reporter assay were performed to examine the activation of NF-κB pathway. Cells were grown in wells of 6-wells plate and co-transfected with specified reporter plasmids and PCMV-β- galactosidase following the transfection process referred in 2.1.5. Treatment may followed in some experiments when stated. Medium was then removed and washed cells with pre-cold 1 x PBS. Directly added 250ul 1 x Cell Culture Lysis Buffer (Promega, E153A) into the well and placed the plate on a room temperature shaker to solve the cells for 20 minutes. After incubation, completely detached cells by using disposable cell scraper

and decanted all 250ul 1 x cell lysis buffer plus cells into a 1.5ml microcentrifuge tube. Vigorously mixed it by vortex for 15 seconds. Centrifuged at 13000 rpm for 1 minute at room temperature to collect supernatant in a fresh tube for analysis. The expression of reporter-driven luciferase was detected by using Luciferase Assay System (Promega, E1500) in the luminometer (Lumat LB 9507 Luminometer, Berthold Technologies). Relative Luciferase Unit (RLU) were output as the indication of luciferase intensity of the sample. 20ul of sample were needed in a 5ml Sarstedt PS tubes (SARSTEDT, 55.1579, Germany) for each measurement. Each sample was measured twice. PCMV-driven expression of β-galactosidase was used as control for transfection and can be detected by using β-Galactosidase Enzyme Assay System (Promega, E2000). β-galactosidase was performed in a 96-wells plate. In each well, thoroughly mixed 50ul sample with 50ul 2x Assay Buffer (Promega, E203A). Each sample was measured three times. Incubated plate at room temperature until the colour of sample solution began to turn pale yellow. Read the plate on a plate reader (Labsystems Multiskan MS, LabX) which output the absorbance at 420nm as relative β-galactosidase intensity. The absolute number of the relative β-galactosidase intensity should below 0.8-1.0 when the sample and 2 x assay buffer reaction came to saturation and the absorbance at 420nm became non-linear.

Data analysis: Analysed relative luciferase activity in Microsoft Excel. Divide the results from the luciferase assay by the results from the galactosidase assay to normalize reporter activity by transfection efficiency. Mean values (added up all values and then divided by the number of repeats) are represented in graphs where there are more than one values. Standard deviations (SD) are also calculated for samples where there are more than one values.

2.6.2 Apoptosis assay

Apoptosis Detection Kit (Calbiochem) was used to detect apoptotic cells by staining cell apoptosis marker, cell surface phosphatidylserine. Manufacturer's instructions were followed and optimized as following. Cells were cultured in T25 flasks and transfected or treated as indicated. Post treatment, medium with floating cells were decanted into a 15 ml Falcon tube at 4°C in order to collect any detectable apoptotic

cells. Washed the attached cells twice with pre-cold 1 x PBS in prior to incubate with 1:1 tyrpsin:versene (T:V) at 37 °C for 5 minutes or until cells detached. 3ml of pre-cold 1 x PBS was then added and moderately pipetted to mix and single the cells before decanting to the 15ml Falcon tube containing floating cells. Counted cells in the Coulter Counter as described above. Collected cell pellets by centrifuging at 2000rpm for 5 minutes and re-suspended at a concentration of $1x10^6$ cells/ ml with appropriate volume of pre-cold 1 x PBS. 1ml of cells ($1x10^6$ cells) were transferred to a microcentrifuge tube and pelleted at 2500 rpm for 3 minutes in 4°C. Re-suspended pellets in 1 x binding buffer (dilute from 5 x binding buffer supplied in the kit) before incubating with 2ul Annexin-V-FITC in darkness at room temperature for 30 minutes and pelleted again with centrifugation at 1000G for 5 minutes. Aliquoted cells into a microcentrifuge tube and incubated as above without adding Annexi-V-FITC to act as a negative control and help analysis parameter setting. Re-suspended pellets in 600ul 1 x binding buffer and placed samples on ice for further analysis.

Detect apoptotic cells by fluorescent microscope

5ul of Vectashield containing 1ug/ml DAPI and 5ul of cells suspension were mixed on a glass slide and mounted with coverslip. Fluorescence microscope (as described in 2.5.2) was applied to count cells with DAPI staining (nuclear) and FITC staining (cell surface phosphatidylserine, looks like a ring under microscope). The percentage of cells undergoing apoptosis was calculated by subtracting FITC-positive cells (apoptotic cells) with DAPI positive cells (total cell number). A minimum of 200 cells were measured per sample.

Detect apoptotic cells by Fluorescence Activated Cell Sorting (FACS)

10ul Propidium Iodide were added into cell suspension in darkness in prior to FACS measurement. BD FACSAriaII SORP (Beckton Dickinson, Oxford, UK) flow cytometer were used. 488 nm laser were used to measure DAPI (685/35 nm bandpass filter) and FITC fluorescence (525/50 nm bandpass filter). The percentage of cells undergoing apoptosis was output. A minimum of 10,000 cells were measured per sample and analysis carried out in BD FACSDiva (Beckton Dickinson, Version 6.1.2) software.

2.6.3 UV-C exposure

UV-C exposure was performed in Hoefer UVC 500 Crosslinker (Hoefer, Inc, USA). Cells were seeded in 6-wells plate (1 x 10^5/cm^2) and grown for 24 hours. Collected medium into 50 ml Falcon tube and placed in 37°C water bath. Washed cells with pre-warmed 1 x PBS once and put another 1ml pre-warmed 1 x PBS in wells to avoid cells dried out. Took of the plate cover and horizontally placed plate into Hoefer UV 500 Crosslinker, and set up intensity of UV-C (254nm) to 40j/m^2. Kept control 'mock' plate in room temperature without exposing to UV-C. Post UV-C exposure, immediately replaced PBS with the pre-warmed collected-medium in 50ml Falcon tube before returning plates into 37°C incubator. Detached and harvested cells into a 15ml Falcon tube after 1 x PBS washing at the indicated time point. Cell pellets were then processed to 'Soluble protein extraction from whole cells' and 'Western blot analysis' as described in 2.4.1 and 2.4.6 respectively.

Chapter 3: Results—Nucleolar stress activates the NF-κB pathway

3.1 Introduction

Although the primary function of nucleolus is ribosome biogenesis, many proteomic and functional studies in the last decade have revealed that the organelle is multifunctional and provides a critical link between stress signalling, ribosome subunit biosynthesis and multiple cellular functions like cell cycle progression and cell proliferation (Boulon et al., 2010).

The most established downstream effector of nucleolar stress is p53 (see section 1.4.1). However, there is increasing evidence to show that nucleolar stress can modulate cell phenotype in a p53 independent manner. For example, recent studies have identified transcription factor E2F1 as regulator of nucleolar stress-mediated cell cycle arrest (Donati et al., 2011); Peter Pan, which mainly localizes in nucleoli and functions in ribosome maturation, links a nucleolar stress response pathway to cell apoptosis (Pfister et al., 2015). Besides, quantitative proteomic analysis of nucleoli in mammalian cells have revealed hundreds of proteins with p53 independent functions shuttle from nucleoli in response to stresses such as UV light, ionizing radiation and DNA damage (Boisvert et al., 2010; Moore et al., 2011). As mentioned in section 1.3, ribosome biogenesis is hyperactivated in the majority of tumours and thus, the organelle is emerging as a promising therapeutic target. Since more than 50% of cancers have non-functional p53, clarifying these p53-independent pathways would allow targeting of nucleoli in p53-deficient cancers and reveal additional molecular targets for cancer therapies that exploit nucleolar stress response pathways (James et al., 2014).

Like p53, the NF-κB pathway is a critical regulator of cell stress (see section 1.5.2) and multiple lines of evidence suggest that, also like p53, NF-κB may act as a downstream effector of nucleolar stress reponse. Firstly, NF-κB is a pleiotropic transcription factor and involved in multiple cellular functions. It regulates the expression of a wide range of genes which are involved in all downstream consequences of nucleolar stress such as cell cycle, apoptosis and senescence.

Secondly, multiple nucleolar proteins and known pathways which are involved in nucleolar stress response are also known in coordinating with or regulating NF-κB

signalling. One of the examples is the protein kinase casein kinase 2 (CK2), a component of Pol I transcription machinery. In this instance, CK2 was shown required for C-terminal phosphorylation of IκBα under UV light stress (Kato et al., 2003). On the other hand, proteins in NF-κB pathway are also evidenced have nucleolar localization or nucleolar function. Such as nucleolar translocation of RelA in response to aspirin or other NSAIDs (Thoms et al., 2007a), and NIK is shuttling between cytoplasm, nucleoplasm and nucleolus and differentially regulates NF-κB pathway (Birbach et al., 2004). In addition, signalling pathways like p53/MDM2, p14ARF, AMPK and p38 MAPK, which are engaged in nucleolar stress response (James et al., 2014), are also known having intriguing inter-connections between NF-κB signalling in multiple situations (Boulon et al., 2010).

Thirdly, the nucleolus and NF-κB are both emerging as critical stress sensors. From literature reviews, it is very striking and interesting to find an extensive overlap between stress inducers that disrupt nucleoli and stimulate NF-κB (Table 3.1). For example, genotoxic stress that causes DNA damage (such as UV light, ionizing radiation, camptothecin), which were shown have exclusive impact on nucleolar proteome and function (Boisvert et al., 2010; Moore et al., 2011), also have long-known effects on NF-κB activation (Leung and Lamond, 2003). Similarly, energy homeostasis status changes induced by oxidative stress/hypoxia, nutrient stresse and oncogenic stress also have simultaneous effects on nucleolar disruption and NF-κB regulation. Most importantly, many chemotherapeutic agents have effects on both the NF-κB pathway and nucleoli, suggesting the exciting possibility that they may be linked.

From the above aspects, the host laboratory had tried to move forward by identifying the molecular network that links modulation of NF-κB and changes in nucleoli in response to aspirin treatment (see Figure 1.10 for preliminary data), which could shed lights on understanding the anti-tumour effect of this agent and identify further targets. Based on these data, I set out to investigate the relationship between nucleolar stress and activation of the NF-κB pathway. Firstly, I examined whether direct disruption of Pol I transcription machinery, could induce activation of NF-κB pathway and alter expression of NF-κB target gene, which is the focus of this chapter.

Stress type	Stimulus	Activation NF-κB	Reference	Nucleolar disruption	Reference
DNA damage/ genotoxic stress	UV-C	y	Wu and Miyamoto, 2007;Li et al, 1998;	y	Al-Baker et al, 2004; Moore et al, 2011, Lamond, 2010; Kruklak et al, 2007; Rubbi and Milner , 2003
	IR (DSB)	y		y	
	Camptothecin	y	Piret et al, 1996, Wu and Miyamoto,	y	
	Bleomycin	y		y	
Oxidadtive stress	ROI	y	Schreck et al, 2001	y	Wang et al, 2012; Mekhail et al, 2003; Rubbie and Milner, 2003
	Hypoxia	y	Kenneth and Rocha, 2008	y	
Chemo-therapeutic /-preventative agents	Daunorubicin/ Doxorubicin	y	Bian et al, 2001;Campbell et al, 2006	y	Burger et al, 2010; Chan et al, 2007
	Etoposide	y	Tabata et al, 2001; Piret et al, 1996	y	Boisvert et al, 2010
	Aspirin/ NSAIDs	y	Stark et al, 2001; Loveridge et al, 2008	y	Stark and Dunlop, 2005; Loveridge et al, 2008
Nutrient stress	Serum starvation	y	Mogi et al, 2004;Stark and Dunlop, 2005	y	Mayer and Grummt, 2006
Oncogenic stress	Ras/BCR-ABL	y	Basseres and Baldwin, 2006; Perkins and Gilmore, 2006	y	Rugerro, 2012; Suzuki et al, 2012

Table 3.1 Stresses that activate NF-κB cause nucleolar disruption (Table made by Dr. Lesley Stark).

3.2 Results

3.2.1 Pol I complex disruption induces NF-κB-driven transcription

Previous studies from the host laboratory suggested that nucleolar stress may act as a common stress sensor which converges on NF-κB signalling. However, this hypothesis was incompletely tested and the potential mechanism that links nucleolar stress to NF-κB signalling is unknown. Therefore, I firstly set out to test whether nucleolar disruption has direct effects on NF-κB activation.

Firstly, I mimicked nucleolar stress in SW480 colon cancer cells using RNA interference against TIF-IA and UBF, two critical components of the Pol I transcription machinery that coordinate rDNA transcription (Drygin et al., 2010). I found that in SW480 cells, knocking down TIF-IA and UBF both efficiently mimicked nucleolar stress as evidenced by the repression of rDNA transcription (Figure 3.1 A). To test the effects of disrupting Pol I on NF-κB activity, NF-κB reporter assays were performed on cells depleted for TIF-IA and UBF. NF-κB reporter constructs in which the luciferase gene is driven by 3x enhancer κB binding

sites, or equivalent plasmids with the κB sites deleted were utilised. TNFα, a strong stimulant of the NF-κB canonical pathway, was used as a positive control (Figure 3.1 B). I found that knocking down TIF-IA and UBF enhanced NF-κB-driven transcription. This was specific as it was not observed when κB binding sites were deleted (Figure 3.1 C and D). This is in agreement with the preliminary data from others in the lab which demonstrated that knocking down Pol I factors induces the degradation of IκBα protein and the phosphorylation of RelA on serine 536, direct signs of NF-κB activation.

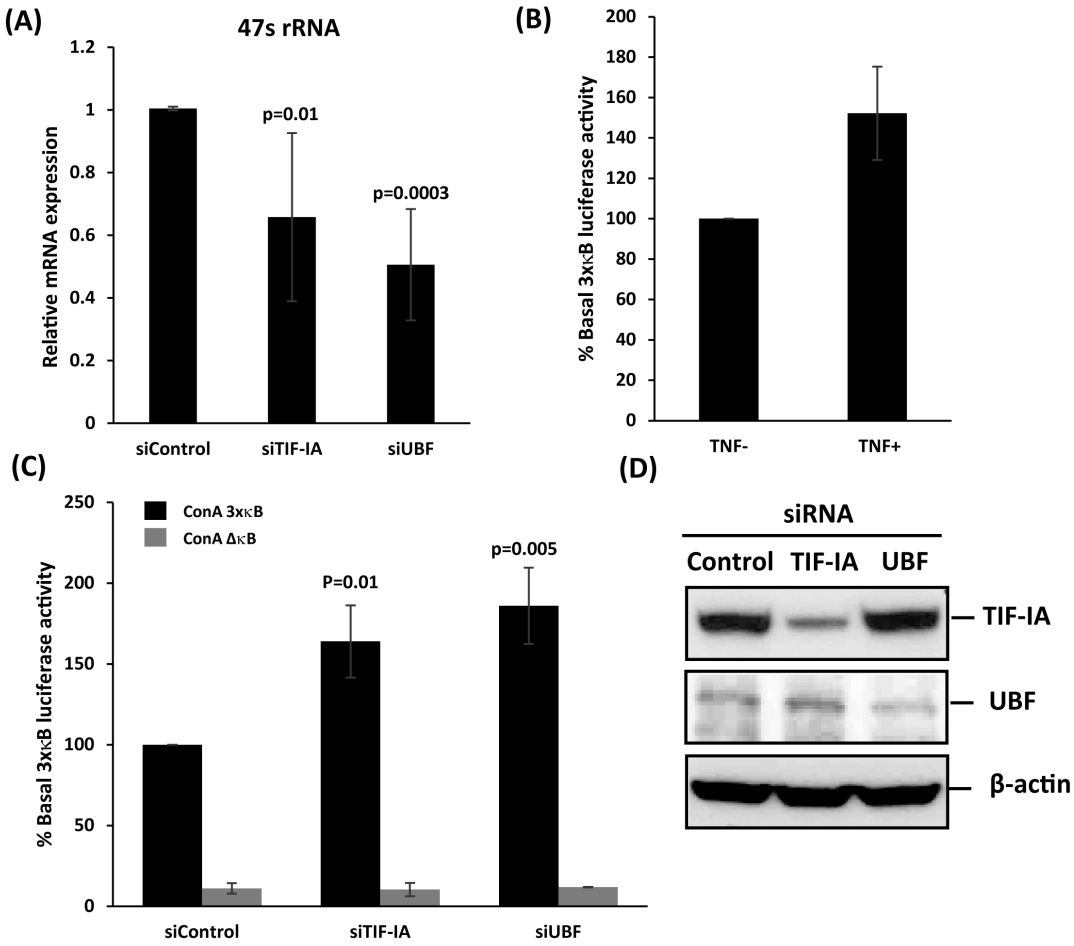


Figure 3.1 Disruption of Pol I complex components activates NF-κB pathway.

(A) SW480 cells were transfected with indicated siRNA. Cells were harvested 48 hours after transfection and total RNA were extracted to synthesis cDNA. qRT-PCR were performed by using Taqman-gene expression system with primers for 47s rRNA. gapdh primer was used as normalization control. Graph shows level of normalised 47s rRNA transcript relative to control siRNA (+/- SD) (calculated using ddCt algorithm). N=5 independent experiments. **(B)** SW480 cells were transfected with 3 ug of wild-type (3x κB ConA) NF-κB dependent luciferase reporter constructs along with 1.5 ug of pCMV-β control plasmid. 24 hours after transfection, cells were exposed to TNFα (10ng/ml for 2 hours). NF-κB activity was determined by relative luciferase activity, results were normalized by β-galactosidase activity and are presented as the percentage relative luciferase activity compared to non-treated control (+/- SD). N=3 independent experiments. **(C)** SW480 cells were transfected with the indicated siRNA species along with 1.5 ug pCMV-β and either 3ug ConA 3x κB-Luc or the control ConA ΔκB-Luc (with κB sites deleted). NF-κB activity was determined by % relative luciferase activity compared to cells transfected with control siRNA (+/- SD). N=3 independent experiments. **(D)** Western blot analysis shows the whole cell lysate protein levels of TIF-IA, UBF or phosphorylated IκBα in cells transfected with indicated siRNA. β-catin acts as protein loading control. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to siControl.

Data above indicated that disruption of the Pol I complex activates the NF-κB pathway. To confirm that knockdown of Pol I factors stimulated cytoplasmic to

nuclear translocation of NF-κB, (rather than affecting DNA bound NF-κB), I used HRT18 cells which the group previously engineered to constitutively express a superrepressor form of IκB (IκBSR) (HRT18-IκBSR). This IκB is mutated at the critical serine phosphorylation sites (S32/S36) and is resistant to TNFα-induced phosphorylation and degradation (Stark et al., 2001; Zwacka et al., 2000). Firstly, I confirmed the expression of IκBSR in HRT18 cells efficiently inhibited TNFα-induced NF-κB-driven transcription activity (about 2-fold decrease compared to HRT18 parental cells) (Figure 3.2 A). Similar to SW480 cells, I found that knocking down UBF and TIF-IA significantly enhanced NF-κB activity in HRT18 parental cells. However, this increase was blocked in HRT18-IκBSR cells (Figure 3.2 B and C).

These results demonstrated that mimicking nucleolar stress by depleting crucial Pol I factors stimulates NF-κB transcriptional activity. They also suggest that cytoplasmic to nuclear translocation of NF-κB is essential for this activation.

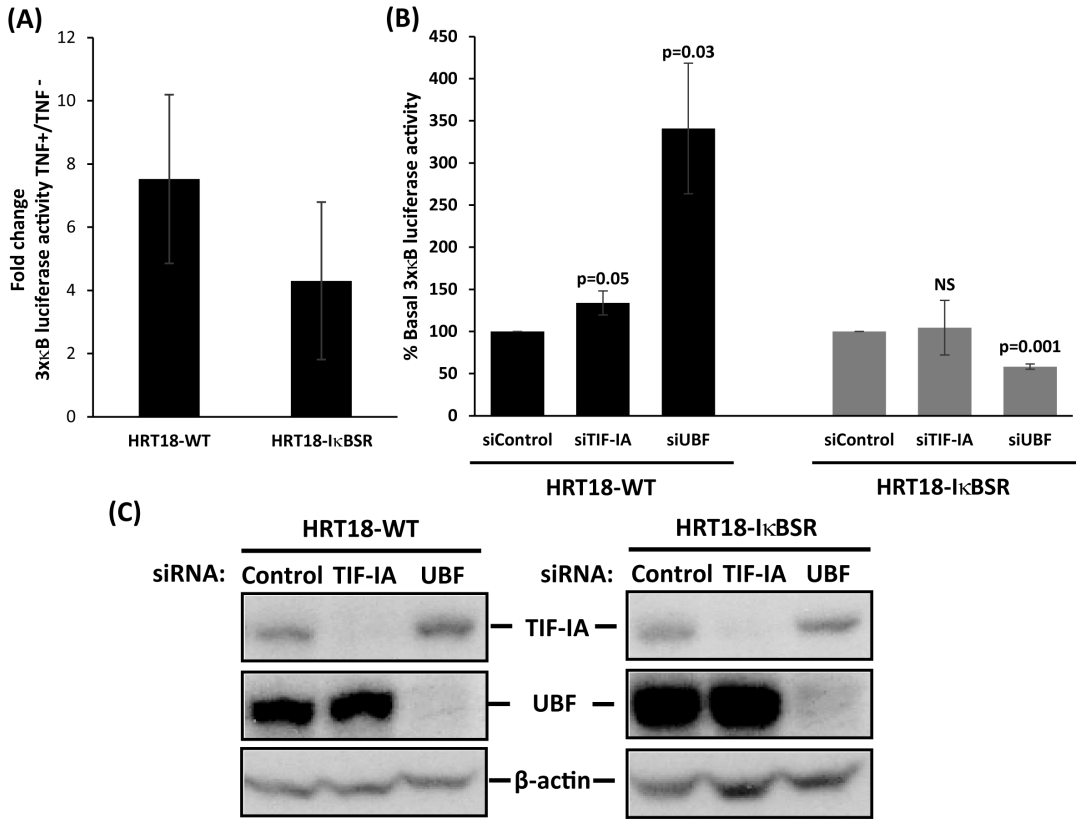


Figure 3.2 Nucleolar disruption activates cytoplasmic NF-κB signalling.

(A) HRT18 parental cells and HRT18-IκBSR cells were transfected with 6 ug 3x κB ConA-Luc along with 3 ug pCMVβ control plasmid 24 hours in prior to TNF treatment (10ng/ml) for 4 hours. Relative luciferase activities are normalized by β-galactosidase activity. Fold change is shown by comparing relative luciferase activity in non-treated and TNF treated samples (+/- SD). N=3 independent experiments. **(B)** HRT18 parental cells and the HRT18-IκBSR cells were co-transfected with the indicated siRNAs, pCMV-β and either 3x κB ConA-Luc or the control ConA ΔκB-Luc. Relative luciferase activity was determined as above and comparted to siControl transfected cells (+/-SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to siControl. **(C)** siRNA transfection efficiency in above graph was tested by western blotting with antibodies against TIF-IA and UBF. β-actin acts as a loading control.

3.2.2 Nucleolar disruption modulates NF-κB-controlled gene expression

Activation of NF-κB will generally lead to increased expression of NF-κB target genes. Therefore, I further determined whether nucleolar disruption activates NF-κB activity by testing effects on the expression of NF-κB target genes. The most recognised and classic target of NF-κB is its inhibitor, IκBα. The Bcl-2 family are also direct transcriptional targets of NF-κB. Western blot analysis indicated that knocking down TIF-IA and UBF in SW480 cells increased cellular levels of Bcl-xL

and IκBα (Figure 3.3 A). Reporter assays, in which the luciferase gene is driven by the full length IκB promotor (kindly supplied by Neil Perkins in the Newcastle University), also indicated that nucleolar disruption significantly increased transcription of this protein. I also indicated this was dependent on NF-κB as the effects were lost when a promotor in which the κB sites were deleted was used. (Figure 3.3 B). Furhtermore, qRT-PCR also suggested depleting TIF-IA or UBF increased *IκBα* gene transcription. These data indicates nucleolar disruption modulates the NF-κB activity and has downstream effects on NF-κB targets (Figure 3.3 C).

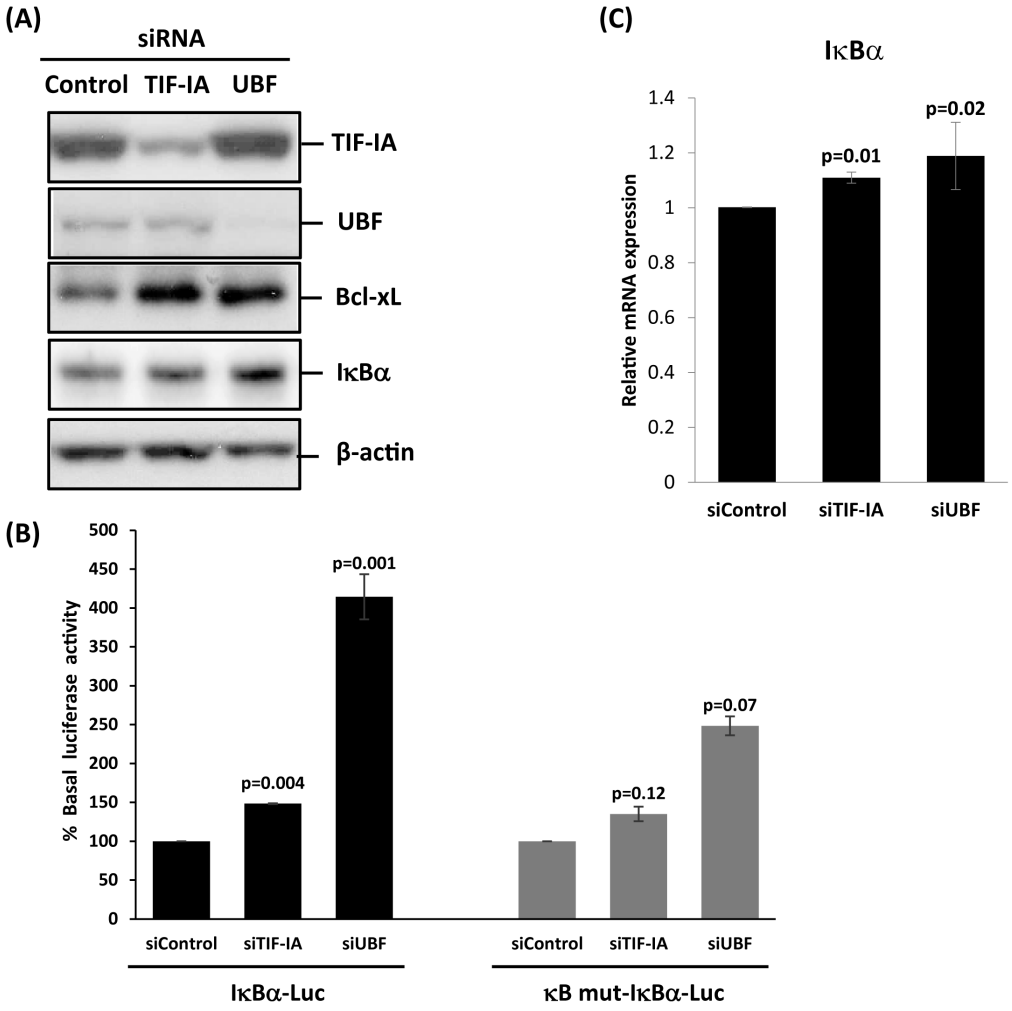


Figure 3.3 Depletion of Pol I factors promotes expression of NF-κB targeted IκBα and Bcl-xL.

(A) Western blotting shows the whole cell lysate protein level of TIF-IA, UBF, Bcl-xL and IκBα in SW480 cells. β-actin acts as a loading control. **(B)** SW480 cells were transfected with indicated siRNA species along with IκB-luc (luciferase driven by full length IκB promoter) or ΔκB-IκB-luc (equivalent in which NF-κB binding sites are mutated) and pCMV-β. Graph depicts the percentage relative luciferase activity compared control siRNA (+/- SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical analysis. **(C)** SW480 cells were transfected with siRNA against TIF-IA, UBF or a scrambled sequence. Cells were harvested 48 hours after transfection. Total RNA were extracted to synthesis cDNA. qRT-PCR were performed by using Taqman gene expression system with primers set for IκBα. gapdh primer was used as normalization control. The graphs depict relative amount of mRNA transcripts comparing to the cells transfected with control siRNA (calculated using ddCt algorithm) (+/- SD). N=5 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to siControl.

The NF-κB pathway is crucial in both inflammatory response and cancer and regulates hundreds of genes involved in these functions. Therefore, I next used qRT-PCR to examine more generally the effects of disrupting the Pol I complex on NF-κB-controlled gene expression. Taqman or SYBR green gene expression assays were

used. I chose several NF-κB target genes to test including *Bcl-xL, BAX and XIAP,* which regulate apoptosis (Figure 3.4 A) and *IL-6, IL-8* and *IL-11* which control inflammatory response (Figure 3.4 B)). It was thought this assay could reveal the cellular functions nucleolar stress directly affects through the NF-κB pathway. Interestingly, I found that most of the NF-κB target genes I tested were significantly repressed following nucleolar disruption (Figure 3.4). They also appeared to fall into two groups. Those that were repressed by both TIF-IA and UBF knockdown (*BAX, IL-6, IL-8*), and those that were only repressed upon depletion of TIF-IA (*Bcl-xL and XIAP*) (Figure 3.4). This result suggested nucleolar stress down-regulates genes involved in inflammatory response that may repress immune response. But how activated NF-κB-driven transcriptional activity upon nucleolar stress leads to repression of NF-κB-targeted genes expression is unclear.

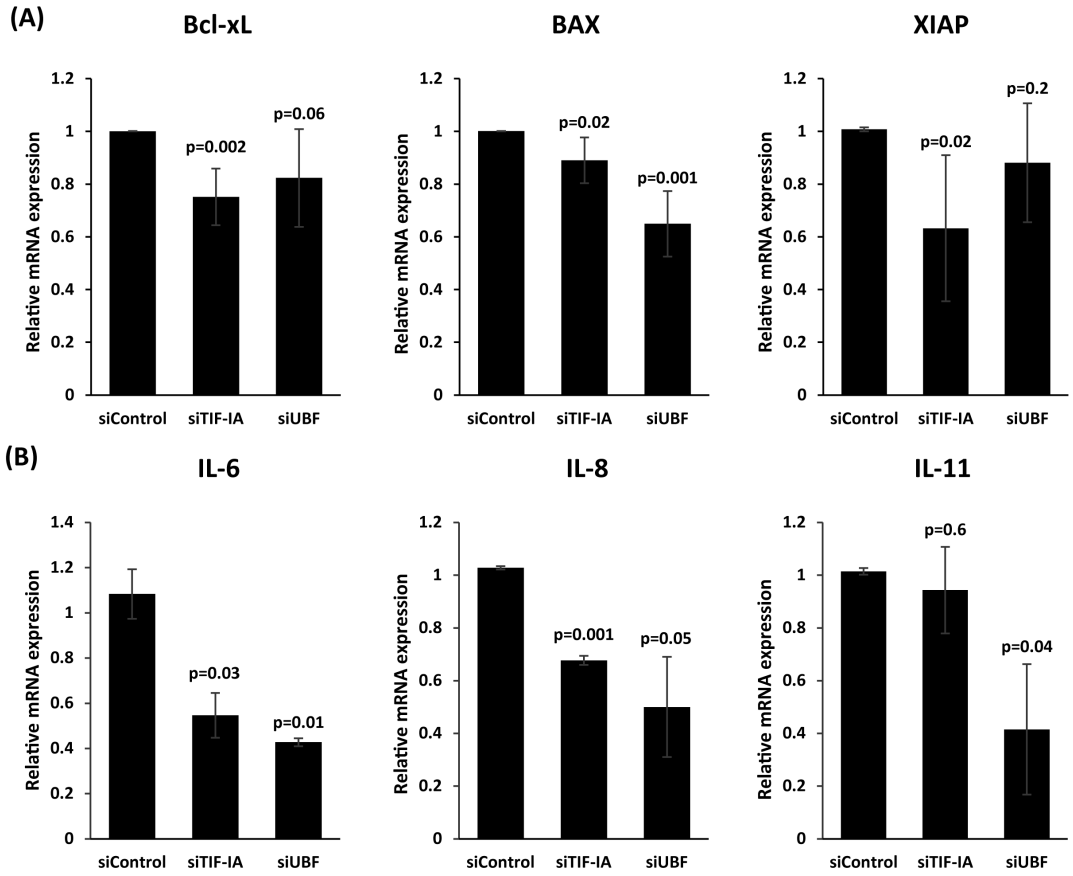


Figure 3.4 Knocking down Pol I factors modulates NF-κB controlled gene expression.

(A and B) SW480 cells were transfected with siRNA against TIF-IA, UBF or a scrambled sequence. Cells were harvested 48 hours after transfection. Total RNA were extracted to synthesis cDNA. qRT-PCR were performed by using Taqman (A) or SYBR green (B) gene expression system with primers set for indicated genes. gapdh primer was used as normalization control. The graphs depict relative amount of mRNA transcripts comparing to the cells transfected with control siRNA (calculated using ddCt algorithm) (+/- SD). N=5 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to siControl.

One explanation for these results may be that in SW480 cells, the genes tested are under control of another pathways. To test this hypothesis, I further examined gene expressions in response to TNFα induction in SW480 cells. Among five NF-κB target genes tested, only the expressions of *IκBα* and *FAS* genes were found to increase in response to TNFα induction (Figure 3.5 A), which suggested in SW480 cells these two genes are direct targets of the NF-κB pathway.

Previous studies had shown that NF-κB dependent gene expression dynamically changes in response to stimuli due to the periodical oscillation of NF-κB between the cytoplasm and nucleus. It was also suggested tha the functional consequence of NF-κB activation may be dependent on the oscillation pattern (Nelson et al., 2004).

Therefore, I performed a siRNA time course experiment to examine the dynamic change of NF-κB-controlled gene expression following nucleolar disruption. I found TIF-IA knockdown induced and increase in *IκBα* gene expression in a time dependent manner. In contrast, UBF knockdown induced an up-and-down pattern with a significant increase at 24 and 48 hours, but a decrease after 56 hours of transfection (Figure 3.5 B). Since IκBα is an inhibitor of NF-κB activation, the repressed gene expression of many genes observed in (Figure 3.4) may due to this negative feedback loop (Figure 3.3 A for protein level, Figure 3.5 B for gene transcription)

Fas gene expression increased in a time dependent manner up to 56 hours following transfection, with UBF depletion having a greater effect than TIF-IA depletion (Figure 3.5 C).

Previous results show nucleolar stress caused NF-κB activation is dependent on the phosphorylation and degradation of IκBα, and cytoplasmic to nuclear translocation of NF-κB. Here I further evidenced NF-κB-targeted *IκBα* gene expression was up-regulated by knocking down UBF, but this upregulation was abrogated in cells transiently expressing superrepressor IκBSR (Supplementary Figure 3.1).

Taken together, these results suggested that besides inducing NF-κB driven transcription, nucleolar stress also regulates the transcription of NF-κB controlled genes. However, this effect was dynamic and highly gene dependent. The dynamic nature of these changes may be the result of NF-κB's oscillation properties.

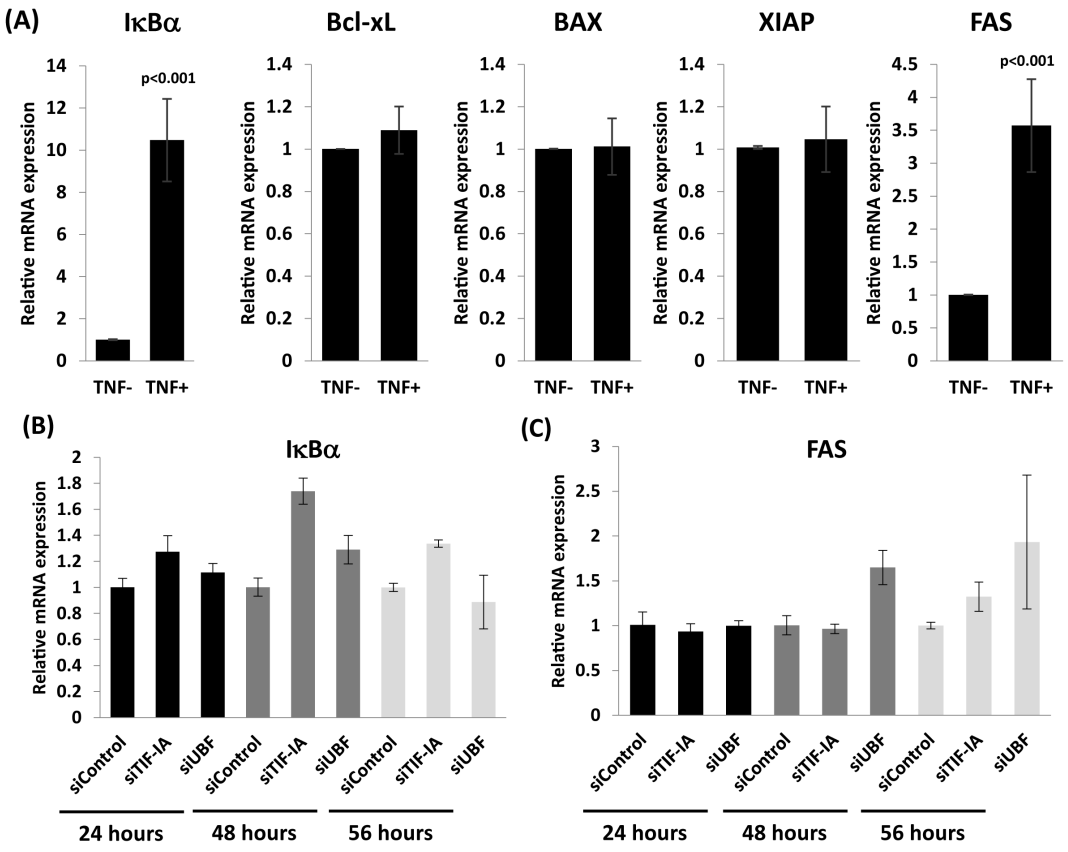


Figure 3.5 Nucleolar disruption-modulated gene expression is time-dependent.

(A) SW480 cells were exposed to TNFα (10ng/ml) for 4 hours before total RNA were extracted to synthesis cDNA. qRT-PCR were performed by using Taqman-gene expression system with primers set for indicated genes. gapdh primer was used as normalization control. Relative level of mRNA transcripts was calculated comparing to the non-treated cells (calculated using ddCt algorithm) (+/- SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to non-treatment control. **(B and C)** SW480 cells were transfected with siRNA against TIF-IA, UBF or a scrambled sequence for indicated time duration. qRT-PCR were performed by using Taqman-gene expression system with primer against IκBα (B) or FAS (C). The graphs show relative amount of mRNA transcripts comparing to the cells transfected with control siRNA (calculated using ddCt algorithm). N=3 technical replicates.

3.2.3 Activation of NF-κB pathway is not a consequence of inhibition of rDNA transcription

The above results indicate that siRNA depletion of TIF-IA and UBF inhibit rDNA transcription and activate NF-κB transcriptional activity, but the mechanism underlying this activation is unknown. Stabilisation of p53 is a common consequence of nucleolar stress and is induced by inhibition of rDNA transcription. To determine whether activation of NF-κB is also caused by inhibition of rDNA transcription, I utilised two novel, highly specific small molecule inhibitors of Pol I, CX-5461 and

BMH-21, which are known to stabilize p53 (Drygin et al., 2011) (Peltonen et al., 2014). I also used low dose of actinomycin D which specifically inhibits Pol I activity. I found these agents, which efficiently and dramatically inhibited rDNA transcription, had no effect on NF-κB driven-transcriptional activity (Figure 3.6 A). This result suggested that, unlike stabilisation of p53, activation of the NF-κB pathway is not a consequence of rDNA transcription inhibition.

3.2.4 CK2 is not involved in Pol I disruption-induced NF-κB activation

From the observations above, it seems NF-κB activation is linked specifically to disruption of the Pol I complex and loss of Pol I integrity. Especially, I assumed this activation may be an 'inside-out' signalling consequence, that disruption of Pol I integrity causes relocation of certain proteins in the complex to the cytoplasm where it stimulates the upstream signalling cascade that culminates in activation of NF-κB. Regarding this hypothesis, the protein kinase casein kinase 2 (CK2) is a favourite candidate. CK2 is one of the factors in the Pol I transcription complex, it reportedly shuttles between the nucleolus, nucleoplasm and cytoplasm, and modulates NF-κB pathway under certain stress condition (Kato et al., 2003). CK2 is a tetramer of two α-subunits and two-β subunits, with the α subunits carrying the catalytic kinase function. I utilised siRNA against CK2 subunits (CK2α and CK2β), and examined their effects on stressed-induced NF-κB activation. I found knocking down CK2 subunits alone repressed NF-κB activity, in keeping with a role in turnover of the protein. Knockdown of UBF had a reduced effect on NF-κB-driven transcription in the absence of CK2, but this was still significant (Figure 3.6 B). Thus, this data suggested CK2 is not involved in Pol I disruption-induced NF-κB activation, although further work is required in this regard.

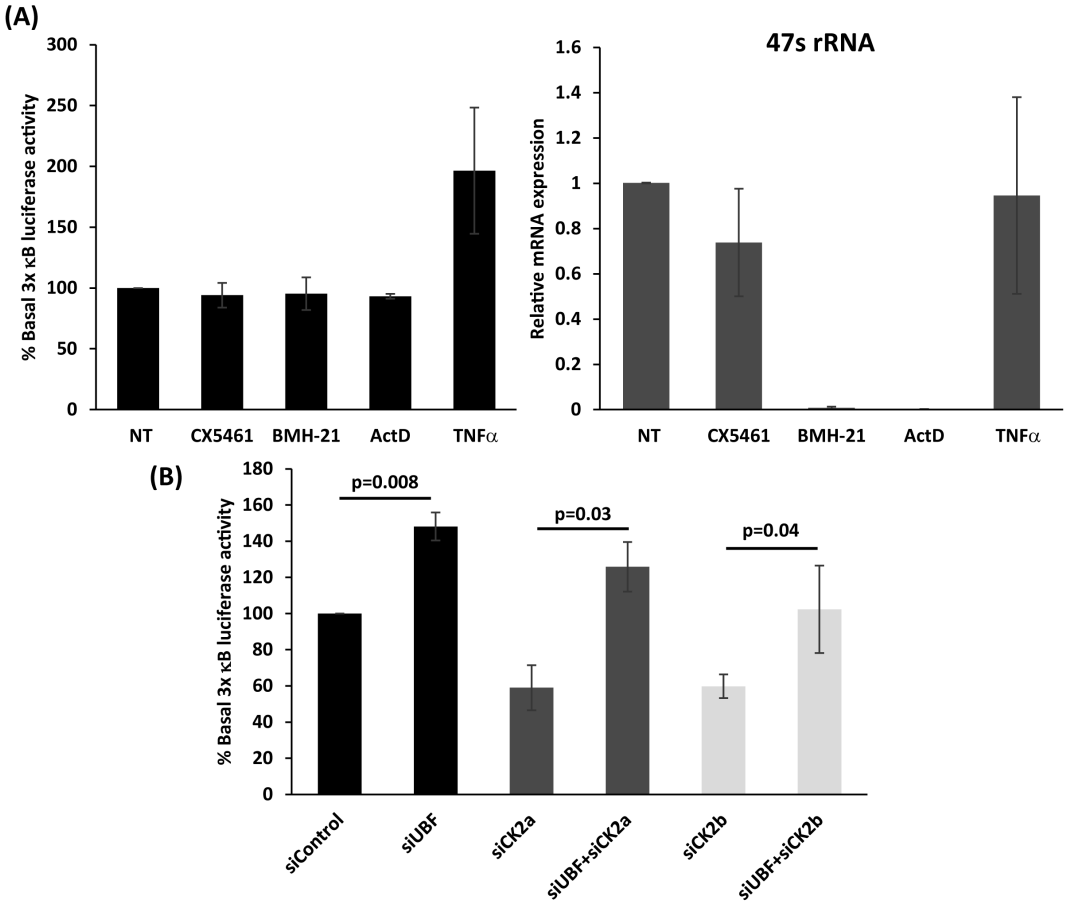


Figure 3.6 Activation of NF-κB pathway is not a consequence of rDNA transcription inhibition and is not dependent on CK2.

(A) *Left.* SW480 cells were transfected with 3ug 3x κB ConA-Luc and 1.5ug pCMVβ. 24 hours later cells were treated with the specified Pol I inhibitors CX5461 (500nM), BMH-21 (4uM), ActD (1ug/ml), or the NF-κB stimulant TNFα (10ng/ml) for 4 hours. Percentage relative luciferase activity was calculated as above, compared to non-treated control (+/- SD). N=2 independent experiments. *Right.* SW480 cells were treated with specified Pol I inhibitors as described above. Total RNA were extracted and subjected to taqman gene expression analysis using primers for 47s rRNA. Relative level of 47s rRNA transcripts were calculated compared to non-treated control (using ddCt algorithm) (+/- SD). N=2 independent experiments. **(B)** SW480 cells were transfected with 3ug 3x κB ConA-Luc and 1.5ug pCMVβ along with indicated siRNA species. 48 hours after transfection, relative luciferase activities were assayed as above, compared to cells transfected with control siRNA (+/- SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical analysis.

3.3 Discussion

In this chapter, I demonstrate that disruption of nucleolar integrity, through artificially knocking down critical Pol I factors, stimulates the cytoplasmic NF-κB signalling pathway and modulates expression of NF-κB targeted genes. I also demonstrated that nucleolar disruption mediated NF-κB activation is not a

consequence of rDNA transcription inhibition, but specifically linked to loss of Pol I integrity. However, factors that link these two events are yet to be identified. This data provides a novel link between nucleolar stress response and modulation of the NF-κB signalling pathway.

NF-κB activation as a downstream consequence of nucleolar stress

So far, the downstream consequences of nucleolar stress are limited to effects on ribosome biosynthesis and stabilisation of p53 (James et al., 2014). Recently studies in models lacking p53 and large-scale nucleolar proteomics strongly suggest the existence of other signalling pathways that regulate cell phenotype as downstream consequences of nucleolar stress. Interestingly, there is a strong correlation between stress inducers that cause nucleolar disruption and that stimulate NF-κB (Table 3.1), but the potential link between these two events has never been investigated.

Here, I made the novel observation that the NF-κB pathway is stimulated by nucleolar disruption. I demonstrate that specifically depleting Pol I factors causes degradation of IκBα, increased NF-κB-driven transcription and modulation on NF-κB controlled gene expression. This observation is further supported by previous findings in the lab that disruption of nucleolar integrity, by means of siRNA interfere against Pol I subunits and expression of site-mutated inactive UBF (Flag-UBF-S388G), enhances phosphorylation of RelA at serine 536 and nuclear translocation of RelA. I also show direct evidence that this activation is dependent on the degradation of IκBα as this effect is lost in cells expressing a superrepressor form of IκB (IκBSR) that retains NF-κB in the cytoplasm (Figure 3.2 B). Furthermore, other members of the lab have observed the same phenomenum in other cell types (e.g HeLa cells), indicating this activation is a general effect and not just limited to colorectal cancer cells.

UBF and TIF-IA are two indispensable Pol I complex components. The importance of them in rDNA transcription and ribosomal biogenesis has been intensively explored and impairment of their activities could lead to rDNA transcription inhibition and nucleolar disruption (Drygin et al., 2010; Grob et al., 2014; Jin and Zhou, 2016). Inhibition of UBF was previously used as a nucleolar stress model in

the very first study demonstrated stabilisation of p53 was the downstream consequences of nucleolar stress (Rubbi and Milner, 2003). In my study, I show siRNA silencing either TIF-IA or UBF suppress rDNA transcription activates the NF-κB pathway and regulates NF-κB target genes. Depletion of UBF had a greater effect than depletion of TIF-IA on most of the NF-κB assays. This is in accordance with the fact that UBF is present on wide ranges of rDNA genes and is involved in multiple steps of rDNA transcription such as initiation, elongation and termination; while TIF-IA is primarily important in rDNA transcription initiation and is mainly present at the active sites of rDNA (Drygin et al., 2010). This further implies the factors that regulate NF-κB activation under Pol I complex disruption is also present at the repressed rDNA.

The molecular mechanism underlying nucleolar stress activation of the NF-κB pathway

Despite my data strongly suggesting that nucleolar disruption stimulates the NF-κB pathway, I have yet to identify the mechanism underlying this activation. However, my data would suggest that this activation is a direct effect of loss nucleolar integrity rather than indirect effects from Pol I transcription inhibition. NF-κB is known to be activated by cell cycle inhibition, which is a consequence of nucleolar disruption (Negi and Brown, 2015; Tsai and Pederson, 2014). However, CX5461 and BMH21, which are known to induce cell cycle arrest (Negi and Brown, 2015; Peltonen et al., 2014), have no effect on the NF-κB pathway (Figure 3.6). This would suggest that in this case, stimulation of NF-κB signalling is not a consequence of the cell cycle inhibition.

It is most likely that Pol I complex disruption causes the loss of nucleolar integrity and relocation of a specific component of Pol I complex or other nucleolar proteins to the cytoplasm where it activates NF-κB signalling pathway. This hypothesis parallels the known RP-Mdm2-p53 ribosome biogenesis surveillance pathway, that is: nucleolar stress causes release of a subset of RPs (for example RPL5 and RPL11) from nucleoli which then bind to MDM2, thus blocking its interaction with p53 and subsequently evoking cell arrest or apoptosis (Deisenroth and Zhang, 2010).

However, I believe the molecular basis underlying nucleolar stress-mediated activation of NF-κB is different from that involved in p53 nucleolar response pathway as I found that Pol I chemical inhibitors (BMH-21 and CX-5461) that stimulate this pathway have no effect on NF-κB-driven transcriptional activity (Colis et al., 2014; Drygin et al., 2011).

The protein kinase casein kinase 2 (CK2) was thought to be a good candidate for linking Pol I complex disruption to NF-κB stimulation. Firstly, CK2 is one of the factors in the Pol I transcription complex (Drygin et al., 2010) and so disruption of the complex could lead to the release of CK2. Secondly, CK2 is known to be modulated by upstream kinase and shuttles between the nucleolus, nucleoplasm and cytoplasm in response to cell stress (Bierhoff et al., 2008). Thirdly, and importantly, CK2 modulates NF-κB pathway upstream of IκBα under certain stress condition (Kato et al., 2003). Therefore, I explored the involvement of CK2 in Pol I disruption-mediated NF-κB by using siRNA against CK2 subunits. However, I found knocking down either of the CK2 subunits had minimal effects on siRNA silencing UBF induced NF-κB activation. One of the shortcomings of this experiment is that CK2 knockdown efficiency was not directly tested by western blot analysis. Further experiments using specific CK2 inhibitors or overexpressing inactive form of CK2 would be desirable to clearly address this question. Another kinase of possible involvement is NF-κB inducing kinase (NIK). NIK is a factor regulating the NF-κB non-canonical pathway, but is known to shuttle between the cytoplasm, nucleoplasm and nucleolus and differentially regulate NF-κB pathway (Birbach et al., 2004).

Downstream effects of nucleolar stress-induced NF-κB activation

NF-κB pathway is a pleotropic signalling pathway that plays a critical role in regulating immune response and cancer development. It targets and controls the expression of a growing list of genes with various functions (website resource: http://www.bu.edu/NF-κB/gene-resources/target-genes/, maintained by Thomas Gilmore's laboratory). In order to understand the downstream consequence on gene expression or cellular functions of nucleolar stress-activated NF-κB, I tested the expression of a set of genes known to be regulated by NF-κB. Consistent with the

finding that nucleolar stress activates the NF-κB pathway, western blot analysis demonstrated Pol I complex disruption increased the protein level of IκBα and Bcl-xL (Figure 3.3A), two proteins whose expressions are directly regulated by NF-κB activation. The effects of nucleolar disruption on gene expression were studied by two methods: gene reporter assay and quantitative RT-PCR. However, these two methods initially demonstrated incompatible outcomes. Gene transcription assays, where luciferase activity was driven by either synthetic κB binding sites or the full-length promotor of the IκB gene showed increased transcription (Figure 3.3 B). In contrast, qRT-PCR demonstrated a significant decrease in gene expression patterns of NF-κB targeted genes under the same conditions (Figure 3.4). There are several hypotheses could explain this conflict.

1) The cell-specific and context dependent activation of NF-κB targeted genes, that is NF-κB may directly regulate the expression of certain genes in other cell types but not or indirectly through cooperating with other signalling pathways in the cell culture system I used. First, in my experimental setting in SW480 cells I found genes known to be regulated by NF-κB, such as *Bcl-xL*, *BAX* and *XIAP*, were not activated by TNFα (a well-known NF-κB stimuli) (Figure 3.5A), suggesting these genes were not direct targets of NF-κB or not only targeted by NF-κB pathway in SW480 cells. Others members in the lab have shown that expression of these NF-κB targets are increased in another colorectal cancer cell line HCT116 following depletion of TIF-IA and UBF, supporting the cell type dependent consequences. Second, NF-κB pathway has complicated inter-talks with other signalling pathways in regulating downstream gene transcription (see section 1.5.1.1). Given the huge change of nucleolar proteome in response to stress (Boulon et al., 2010), it is highly possible that other pathways are also modulated in response to Pol I complex disruption and cooperate with NF-κB to regulate downstream genes.

2) NF-κB mediated gene expression was shown to dynamically change following time after stimuli due to a negative feedback loop driven by NF-κB-regulated IκBα transcription, which caused the periodically oscillation of NF-κB between cytoplasm and nuclear (Nelson et al., 2004). Therefore, the down-regulations of these NF-κB targeted genes I observed in qRT-PCR experiments may just represent the gene

transcription status of a specific timing (48 hours of siRNA transfection). Indeed, Figure 3.5 shows siRNA interfere against TIF-IA or UBF after 48 hours increased the protein level of IκBα, the inhibitor of NF-κB activation, which could be a direct clue explaining the gene expression repression shown in Figure 3.4 in the same experiment setting. Furthermore, to address the dynamic change of NF-κB-regulated gene expression under nucleolar disruption, I performed siRNA time course experiment in prior to qRT-PCR analysis. Observations from this experiment, which shown the transcription of the two NF-κB directly targeted genes *IκBα* and *Fas* were dynamically changed after siRNA silencing of TIF-IA or UBF, supporting the dynamics of gene expression. However, this time-course experiment just done once, and it would be with great interest to examine the dynamic change of other genes under Pol I complex disruption. Moreover, it would be beneficial to generate TIF-IA or UBF conditionally knocking-down cell lines and perform gene microarray in these cells to address this question.

3) NF-κB-responsive gene transcription is known to be repressed by NF-κB repressing factor (NRF) through interacting with specific negative regulatory DNA elements on certain NF-κB targeted genes promoters (Nourbakhsh and Hauser, 1999). Most importantly, NRF is dominantly located in nucleolus and is released to nucleoplasm under stress condition like actinomycin D treatment (Niedick et al., 2004). It would be interesting to investigate whether the location of NRF is changed and expression of NRF is enhanced following Pol I complex disruption.

4) Recent studies have shown RNA-binding protein RC3H1 (or ROQUIN) promotes mRNA degradation of NF-κB pathway regulators and NF-κB transcriptional targets, including TNFα (Leppek et al., 2013), A20 and IκB (Murakawa et al., 2015). RC3H1 targets these transcripts by recognizing a constitutive decay element (CDE) in the 3'UTR and recruiting a CCR4-CAF1-NOT deadenylase complex (Murakawa et al., 2015). Therefore, it would be interesting to know whether RC3H1 also help decay the mRNA transcripts of the genes I tested in Figure 3.4 under nucleolar stress. Notably, studies have already evidenced RC3H1 accumulated in stress granules, which suggested a potential role of RC3H1 in stress response (Athanasopoulos et al., 2010).

One of the initial aims on studying gene expression pattern of NF-κB targeted genes was to understand the phenotypic outcome under Pol I complex disruption and to determine whether NF-κB required for this outcome. The data on inflammatory related gene expression may implicate a repression effect on immune response of nucleolar disruption, but the data I obtained did not give us a clear idea whether cell apoptosis was directly affected under this disruption. In order to clearly understand whether Pol I complex disruption mediated phenotypic outcome is through NF-κB activation, further qRT-PCR should be perfomed by knocking down RelA along with the Pol I factors.

In summary, the overwhelming evidence demonstrated NF-κB activation is a direct downstream consequence of nucleolar disruption. Given the extensive overlap between stimuli that activate NF-κB and those that cause nucleolar disruption (Table 3.1), suggesting a potential central role of nucleolus in linking stress response signalling and downstream NF-κB activation. To further examine this relationship, I set out to determine the effects of stress stimuli of NF-κB on the Pol I complex.

Chapter 4: Results—stress stimuli of NF-κB induce degradation of the Pol I transcription initiation factor, TIF-IA

4.1 Introduction

In the last chapter I demonstrated that the NF-κB pathway is activated by depletion of crucical Pol I factors, a distinct type of nucleolar stress.

Nucleolar stress is a general term for describing modifications in nucleolar structure and function that eventually lead to modulation of cell homeostasis (James et al., 2014). It is recognised that the varied effects of different stresses on nucleolar function induced by different kinds of cellular stresses would lead to different form of nucleolar stress, which, for instance, could be distinguished by distinct nucleolar morphological changes (reviewed in section 1.4).

Previous findings in the lab, aimed at identifying the anti-tumour effects of aspirin, revealed this agent also causes changes to nucleolar morphology in colorectal cancer cells. Interestingly, the observed changes to nucleoli were distinct from other forms of nucleolar stress. That is, aspirin caused an increase in nucleolar size, decreased nucleolar number and formation of 'nucleolar caps' without segregation (that is proteins in FC moved to the periphery, but they were not separated from, and were still surrounded by, the DFC). The lab also demonstrated these distinct morphological changes were paralleled with a reduction in rDNA transcription, which preceded the the effects of the agent on the NF-κB pathway. They also found that other stress stimuli of NF-κB had similar effects on nucleolar morphology suggesting the two events may be linked. However, the molecular mechanism driving this morphological change and whether there is a connection between aspirin (or other stresses) effects on nucleoli and stimulation of NF-κB were unknown. To address this, I next investigated the effects of aspirin as a tool stress stimuli of the NF-κB pathway on componets of the Pol I complex.

As outlined in Chapter 1, components of the Pol I transcription machinery are regulated by various upstream pathways in different conditions, mainly by the means of post-translational modifications (Drygin et al., 2010). Among these components, the regulation of transcriptional initiation factor –IA (TIF-IA) has been extensively studied in response to stress. TIF-IA interacts with PolI β and coordinates the interaction between Pol I SL-1/TIF-IB to recruit Pol I to the rDNA promoter to

assemble the transcription initiation complex. It senses environmental signals, plays an important role in cell growth and death, and also controls nucleolar morphology. The activity of TIF-IA is mainly regulated by its phosphorylation status. There are multiple phosphorylation sites on TIF-IA have been identified regulated by various upstream signalling pathway, such as JNK2, mTOR, AMPK, p38 MAPK, in coordinating growth signals, stress response and rDNA transcription.

Although data on the regulation of TIF-IA protein abundance is not profuse, Fatyol and Grummt have shown TIF-IA is regulated by ubiquitination dependent degradation. They demonstrated 19S proteasome complexes were capable of binding to rDNA and 19S proteasome subunit Sug1, a E3 ligase, recognized and bound to the 94 amino acids on TIF-IA N-terminus thus inducing degradation of this protein (Fatyol and Grummt, 2008). One more study demonstrated that MDM2 acts as an ubiquitin liagse for TIF-IA, and that phosphorylation of TIF-IA by an Akt-CK2 axis prevents MDM2-mediated TIF-IA degradation (Nguyen le and Mitchell, 2013). However, to my best knowledge, the dynamics of TIF-IA protein levels following cellular stress has never been reported.

In this chapter, firstly, I demonstrate that aspirin and other stress stimuli of the NF-κB pathway, such as UV-C and ceramide, cause a reduction in levels of TIF-IA. I characterise the molecular mechanisms underlying this reduction. Based on previous findings in the lab that inhibition of CDK4 mimics aspirin effects on the NF-κB pathway (Thoms et al., 2007b), and data demonstrating CDK4 targets the Pol I complex and regulates rDNA transcription (Voit et al., 1999), I considered that CDK4 inhibition may play a role in this pathway. Indeed, I confirm that inhibiting CDK4 activity mimics stress effects on TIF-IA.

4.2 Results

4.2.1 Aspirin induces a reduction in TIF-IA protein levels

To further understand the connection between stress-induced nucleolar modification and stimulation of NF-κB pathway, I set out to explore the molecular changes to nucleoli in response to stress stimuli of NF-κB. Given the critical role of the Pol I

transcription complex in regulating nucleolar function, especially stress response, I firstly examined the effects of stress on protein levels of Pol I factors.

Western blot analysis performed on whole cell lysates indicated the total level of TIF-IA protein rapidly (after 1 hour) decreases in SW480 colorectal cancer cells following exposure to aspirin. In contrast, the agent had a minimal effect on levels of two other Pol I factors, Pol I subunit RPA194 and UBF, in this time frame (Figure 4.1 A). In addition, pharmacological relevant low concentration of aspirin (<3mM) for two-days treatment displayed the same effects on TIF-IA protein level as the high concentration treatment in a short time period (Figure 4.1 B). I excluded the possibility that this reduction in protein levels observed by western blot analysis was due to the antibody specificity or domain specificity by using two antibodies from different species and recognising different domains of TIF-IA (Figure 4.1 A).

I found that aspirin effects on TIF-IA paralleled effects on nucleolar morphology and rDNA transcription (Figure 1.10), suggesting they may be linked. This link was also supported by the gene silencing experiments in the previous chapter that siRNA against TIF-IA, which caused the reduction in TIF-IA protein level, induced a significant reduction in rDNA transcription (Figure 3.1 A).

Together, these data, suggest aspirin induces a reduction in TIF-IA protein in colorectal cancer cells, and that this reduction may lead to the inhibition of rDNA transcription.

It was reported that stress causes re-location of TIF-IA from the nucleolus to nucleoplasm and that is causally involved in the inhibition of rDNA transcription (Mayer et al., 2005). By immunocytochemistry, I found TIF-IA was present in distinct nucleolar foci before aspirin treatment, reminiscent of fibrillar centres. However, in response to aspirin TIF-IA was dispersed throughout the nucleus and formed ‘nucleolar cap’, which suggested a potential nucleolar fragmentation (Figure 4.1 C and D). This dispersal was consistent when cells were treated with the agent at high dose for a short period or low dose for a longer period.

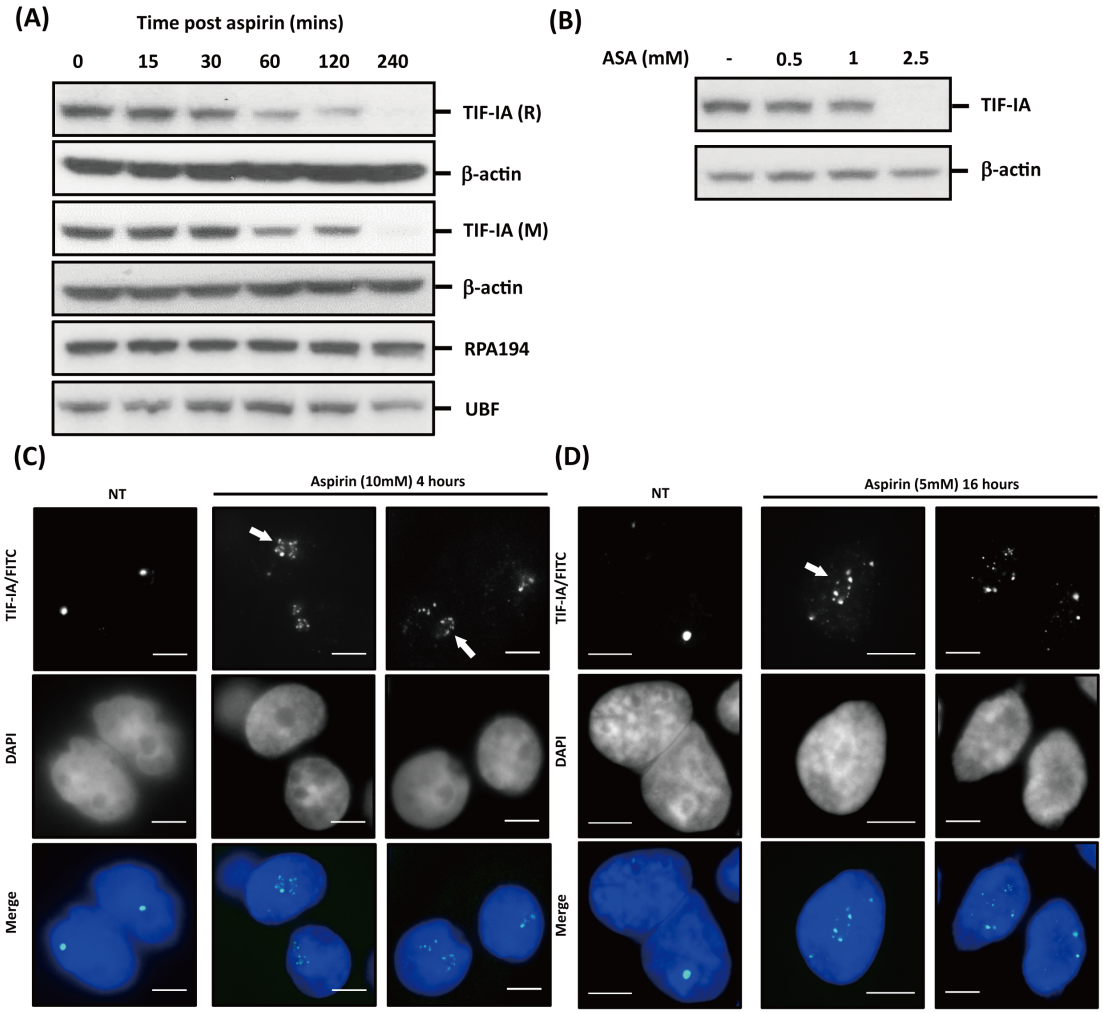


Figure 4.1 Aspirin induces a rapid reduction of TIF-IA protein level.

(A and B) SW480 cells were exposed to 10mM aspirin for the indicated time (A), or 0-2.5mM aspirin for 48 hours (B). Western blotting shows the protein levels of TIF-IA (TIF-IA (R) antibody recognises TIF-IA around phosphorylation site of serine 649 (C terminal); TIF-IA(M) antibody recognises TIF-IA 1-300aa (N-terminal)), RPA194 and UBF. β-actin acts as a loading control. **(C and D)** SW480 cells were exposed to aspirin as indicated conditions. Immunocytochemical analysis shows the cellular location of TIF-IA (FITC, green). DNA was stained by DAPI (blue). Image 63X magnification. Scale bars=10um. Arrows show the 'nucleolar cap' or nuclear dispersion of TIF-IA.

4.2.2 Stress-specificity of effects on TIF-IA

I next explored the generality of TIF-IA depletion with regards to stress stimuli of the NF-κB pathway.

I found UV-C exposure also induced TIF-IA reduction prior to degradation of IκBα (Figure 4.2 A). However, unlike aspirin effects, this depletion was transient and levels were ultimately restored. In contrast to UV-C, the DNA damaging agents

camptothecin and hydrogen peroxide had no effect on TIF-IA levels, and didn't induce IκBα degradation in the indicated time frame (Figure 4.2 B and C).

NF-κB stimuli trigger the generation of ceramide, a crucial lipid second messenger that integrates stress signalling with the induction of apoptosis (Rotolo et al., 2005). Previous results in the lab found that the soluble forms of ceramide, C2 and C6 (which can mimic the endogenous ceramide induction following stresses), induced enlargement and segregation of nucleoli in a similar fashion to aspirin. They also found these effects preceded stimulation of NF-κB signalling and the induction of apoptosis. Therefore, I next examined the effects of C2- and C6- ceramides on protein levels of TIF-IA. Figure 4.2 D indicates that C2 and C6 induce depletion of TIF-IA and that this is not enhanced by concomitant exposure to aspirin (Figure 4.2 D). These data suggest a potential causal link between nucleolar stress, TIF-IA depletion and downstream signalling effects under certain stress conditions.

To determine whether modulation of TIF-IA levels was restricted to NF-κB stimuli, I examined the effects of chemical inhibitors of Pol I transcription, CX-5461, BMH-21 and low dose actinomycin D, on the protein. I found these agents, which had a minimal effect on NF-κB-driven transcription activity, did not cause a reduction in TIF-IA protein (Figure 4.2 E). This further demonstrates specificity and also indicates TIF-IA depletion is not a consequence of rDNA transcription inhibition. Taken together, these data suggest that certain stress stimuli of NF-κB, such as aspirin and UV-C, and the second messenger ceramide induced a reduction in TIF-IA protein levels.

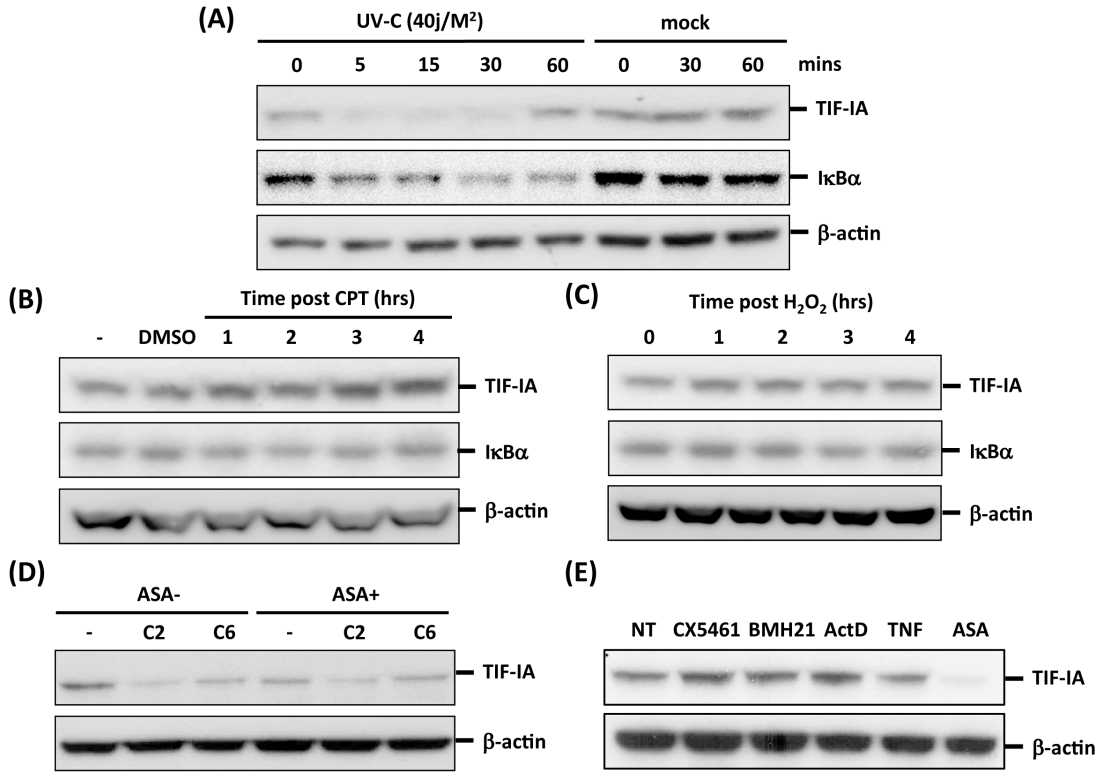


Figure 4.2 Stress stimuli of NF-κB induce TIF-IA depletion.

(A) SW480 cells were exposed to UV light (40j/M^2). Cells were harvested in the indicated time after UV-C exposure or mock. **(B and C)** SW480 cells were treated with DNA damage agents camptothecin (10uM) or hydrogen peroxide (200uM) for the indicated time. **(D)** SW480 cells were treated with ceramide isoform C2 (10uM) or C6 (10uM) for 16 hours. **(E)** SW480 cells were exposed to specific Pol I inhibitors CX5461 (500nM), BMH-21 (4uM), ActD (1ug/ml), or the NF-κB stimulant TNFα (10ng/ml) for 4 hours. Immunoblots show the whole cell lysate protein level of TIF-IA and IκBα. β-actin acts as a loading control.

4.2.3 Aspirin-induced TIF-IA depletion is not through inhibiting *TIF-IA* gene expression

Having identified TIF-IA depletion as a putative cause of the effects of stress stimuli, on nucleolar structural change, rDNA transcription inhibition and downstream stimulation of NF-κB pathway, I wished to elucidate the mechanism involved in this reduction in protein levels in order to inhibit this effect and further establish its role in these downstream events.

Aspirin may block the transcription or translation of the *TIF-IA* gene. To determine whether this is the case, aspirin effects on mRNA levels of *TIF-IA* were examined by RT-PCR and qRT-PCR in kinetic studies. Figure 4.3 A and B show that aspirin has a minimal effect on *TIF-IA* mRNA levels in the time frame during which aspirin

induces TIF-IA protein reduction. To further explore aspirin's effects on TIF-IA translation, I used cyclohexamide as an inhibitor of protein synthesis. SW480 cells, or SW480 cells transfected with exogenous pEGFP-TIF-IA, were exposed to cycloheximide in the presence or absence of aspirin. I found that 8 hours exposure to cyclohexamide had no effect on endogenous or exogenous TIF-IA protein levels, suggesting this protein's half-life in SW480 cells is longer than 8 hours (Figure 4.3 C-F). These data contrast with that reported for U2OS cells, in which de novo synthesised TIF-IA exhibited a half-life of less than 4 hours (Fatyol and Grummt, 2008). In contrast, in the presence of aspirin, TIF-IA protein levels were reduced by 50% in 2 to 4 hours (Figure 4.3 C-F). This result implies that aspirin causes TIF-IA depletion through protein degradation pathways rather than blocking protein synthesis.

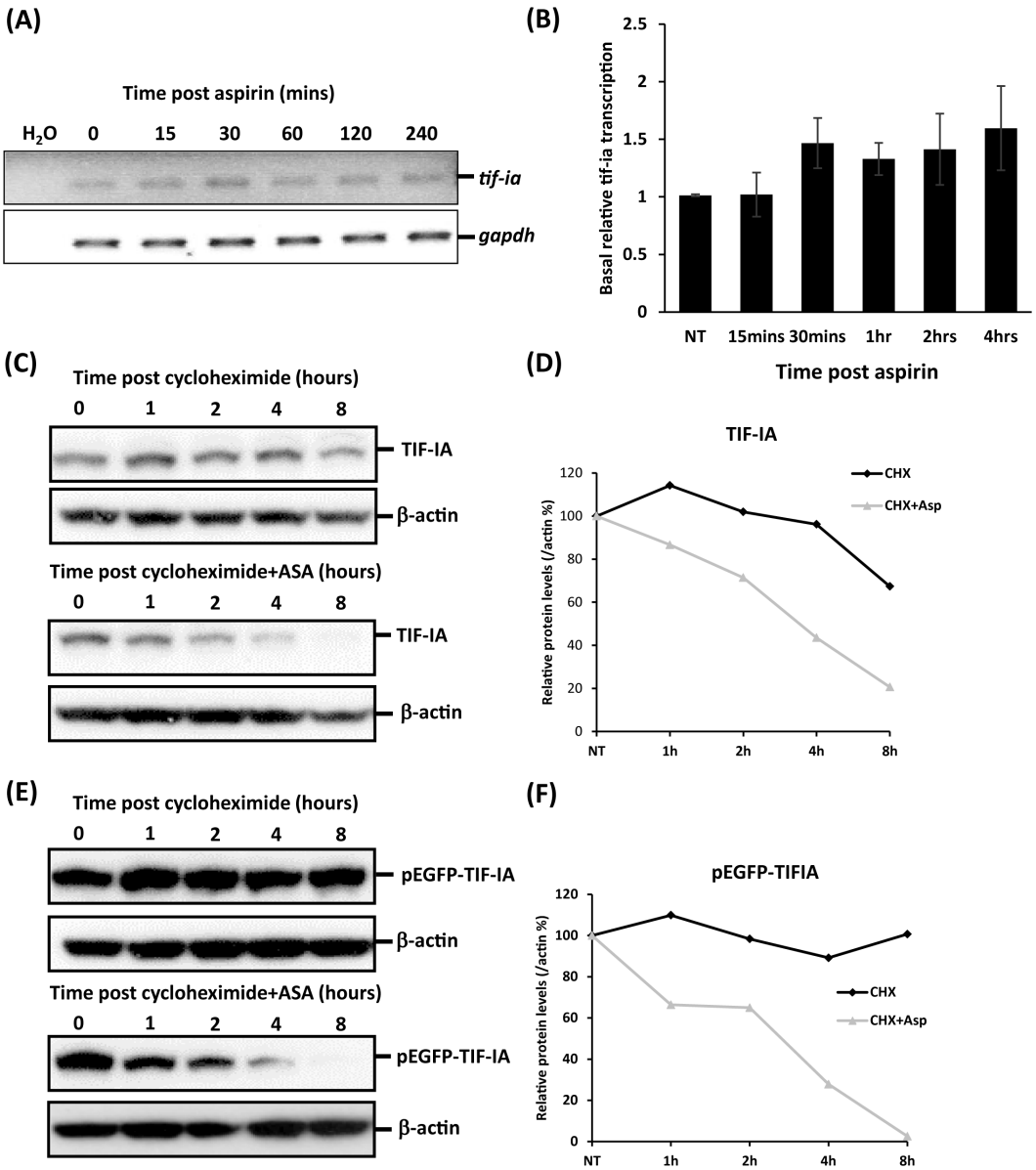


Figure 4.3 Aspirin-induced TIF-IA depletion is not due to transcription or translational inhibition of this protein.

(A) SW480 cells were exposed to aspirin 10mM for the indicated times. Total RNA were extracted and synthesis for cDNA. PCR were performed using primers for TIF-IA. gapdh primer was used as control. PCR products were detected by running 1.5% agarose gel and stained with ethidium bromide. **(B)** The above cDNA samples were analysis by qRT-PCR using SYBR green gene expression system with primers for TIF-IA. gapdh primer was used as control. The relative amount of tif-1a mRNA transcripts were quantified by comparing to the non-treated control (calculated by ddCt algorithm) (+/- SD). N=3 independent experiments. **(C)** SW480 cells were exposed to cycloheximide (10uM), or cycloheximide plus aspirin (10mM) for the times indicated. TIF-IA protein levels were determined by western blot analysis in whole cell lysis. β-actin was used as a loading control; **(E)** *Left.* SW480 cells were transfected with pEGFP-C1-hTIF-IA plasmid. 24 hours after transfection, cells were treated as described in (C). pEGFP-TIF-IA level were detected by antibody for TIF-IA; **(D and F)** Western blot bands from (C) and (E) were quantified by Image J. Results shown are relative intensity of TIF-IA normalized by β-actin.

4.2.4 Aspirin-induced TIF-IA depletion is due to protein degradation

The above data implies that aspirin-induced TIF-IA depletion is through protein degradation. Intracellular protein degradation is mainly executed by two protein catabolism machines: proteasome and lysosome (Hideshima et al., 2005). In addition, two previous studies have shown that endogenous TIF-IA is regulated by proteasome dependent degradation (Fatyol and Grummt, 2008; Nguyen le and Mitchell, 2013).

To explore the role of proteasome-dependent degradation in aspirin-mediated TIF-IA depletion, I used two proteasome inhibitors--MG132 and lactacystin. Although both proteasome inhibitors caused an increase in basal levels of TIF-IA, they did not completely block aspirin-induced depletion of this protein (Figure 4.4 A-C). In contrast, degradation of cyclin D1, an early response of aspirin treatment in colorectal cancer cells (Thoms et al., 2007b), was distinctly blocked by proteasome inhibitors (Figure 4.4 A-C). This result suggests that proteasome dependent degradation regulates the basal levels of TIF-IA as reported (Fatyol and Grummt, 2008), but cannot fully explain aspirin effects on levels of the protein.

Lysosome inhibitors quinacrine and bafilomycin were next employed to test the role of lysosomes in aspirin-induced TIF-IA depletion. Both of the lysosome inhibitors caused increased levels of the control protein, cathepsin D, a lysosomal aspartyl protease (Figure 4.4 D-F). Interestingly, basal levels of TIF-IA were also increased significantly after lysosome inhibition. However, aspirin-mediated depletion of TIF-IA was not fully affected by the presence of these inhibitors (Figure 4.4 D-F).

There are increasing reports of redundancy in degradation pathways in that if one pathway is blocked, the protein is degraded by another (Lilienbaum, 2013). Therefore, I next examined aspirin effects in the presence of inhibitors of both pathways. Interestingly, I found that a combination of both inhibitors significantly abrogate aspirin-mediated TIF-IA depletion (Figure 4.5 A-C).

Taken together, these results suggest aspirin-induced TIF-IA reduction is through promoting protein degradation, mediated by a combination of proteasomal and lysosomal degradation pathways.

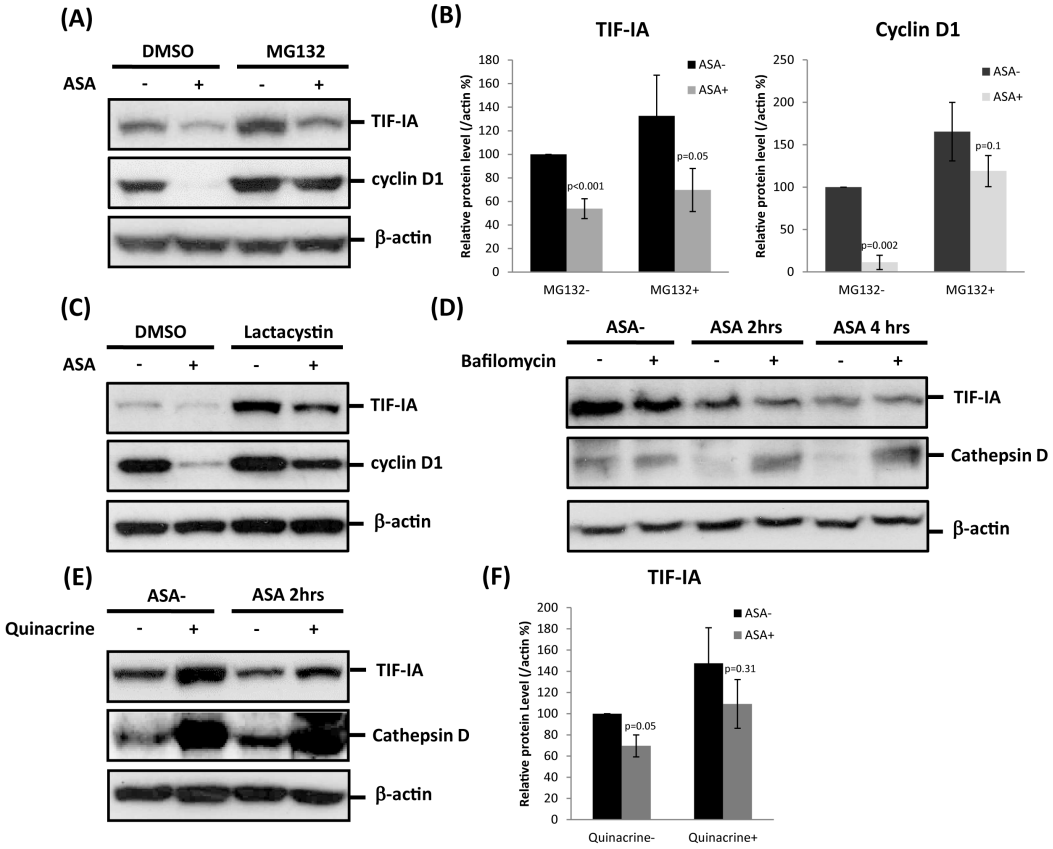


Figure 4.4 Aspirin induced TIF-IA degradation cannot be blocked by separately using proteasomal or lysosomal inhibitors. (A) SW480 cells were pre-treated with MG132 (50uM) for 1 hour in prior to aspirin (10mM, 4 hours) treatment. DMSO was used as vehicle control for MG132. Western blot analysis was performed on whole cell lysates to determine TIF-IA and Cyclin D1 protein levels. **(B)** Western blot bands from (A) were quantified by Image J. Results shown are relative intensity of TIF-IA or Cyclin D1 normalized by β-actin (+/-SD). N=3 independent experiments. **(C)** SW480 cells were pre-treated with lactacystin(10uM) for 24 hours before aspirin(10mM, 4 hours) treatment. DMSO was used as vehicle. Whole cell extracts were harvested and western blotting was performed as above. **(D)** SW480 cells were exposed to bafilomycin (20nM) for 16 hours followed by aspirin (10mM) treatment for 2 hours or 4 hours. Whole cell extracts were prepared and western blot analysis performed to detect TIF-IA and cathepsin D. **(E)** SW480 cells were pre-treated with quinacrine (25uM) for 2 hours in prior to aspirin (10mM, 2 hours) treatment. Anti- TIF-1A and cathepsin D western blot analysis was performed on whole cell extracts. **(F)** Western blot bands from (E) were quantified by Image J. Results shown are relative intensity of TIF-IA normalized by β-actin (+/-SD). N=2 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to ASA-.

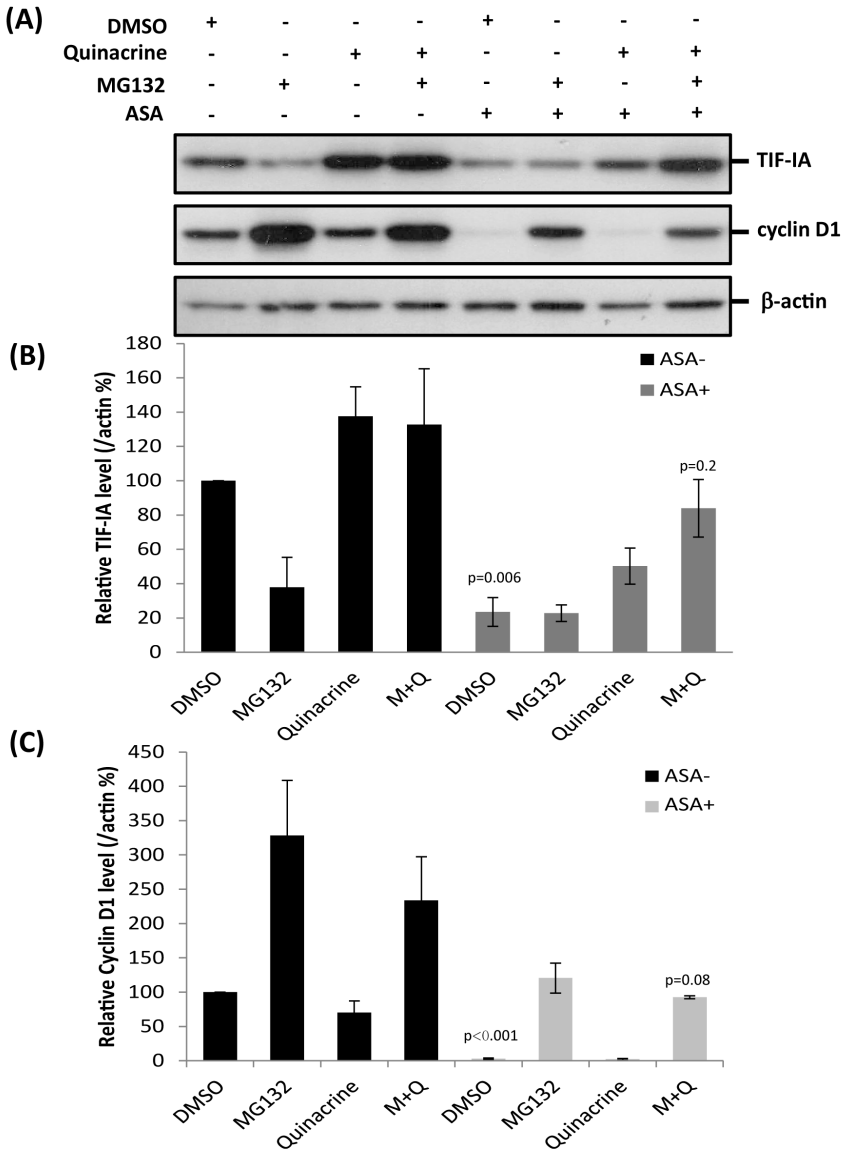


Figure 4.5 Combination of proteasomal and lysosomal inhibitors blocks aspirin effects on TIF-IA.

(A) SW480 cells were pre-treated with quinacrine (25uM), MG132 (25uM) or both for 2 hours prior to aspirin (10mM, 4 hours) treatment. DMSO acts as vehicle control. Anti- TIF-1A and cyclin D1 western blot analysis was performed on whole cell extracts. β-actin was used as a loading control for all experiments settings. **(B and C)** Western blot bands from (A) were quantified by Image J. Results shown are relative intensity of TIF-IA (B) or Cyclin D1 (C) normalized by β-actin (+/-SD). N=2 independent experiments. Two - tailed student's T - test was applied for statistical analysis. p value shows the difference compare to ASA-.

4.2.5 N-terminal 94-204 amino acids of TIF-IA are sufficient for aspirin response

In order to help establish the mechanism by which aspirin affects TIF-IA, I next clarified the domains of TIF-IA required for aspirin-induced depletion. TIF-IA deletion plasmids were generated from pEGFP-C1-hTIF-IA (kindly supplied by Ingrid Grummt, German Cancer Research Centre) (Figure 4.6 A). SW480 cells were transfected with the GFP-tagged constructs then anti-GFP western blot analysis used to examine aspirin effects on levels of the deleted proteins. It demonstrates aspirin induces a significant reduction in levels of all the deletions, even the shortest fragment (94 -204 amino acids) (Figure 4.6 B and C). This suggests that the 94-204 amino acid domain is sufficient for responding to aspirin-mediated stress.

Next I employed immunocytochemistry to understand the cellular location of the deletion proteins. SW480 cells were transfected with the GFP-tagged constructs then GFP localisation determined on fixed cells. As observed in Figure 4.6 D, full-length TIF-IA was predominately located in nucleolus as expected. The C-terminal (403-651 amino acids) of TIF-IA, but not N-terminal (1-94 amino acids), are required for nucleolar localisation, as constructs with only 1-94 amino acids deleted still predominately located in the nucleolus while constructs lacking the C-terminal 250aa were predominantly in the cytoplasm (Figure 4.6 D). These data would suggest the 403-651 amino acids of TIF-IA is critical for the nucleolar localisation of this protein. It also suggests that aspirin-induced TIF-IA degradation takes place in the cytoplasm as the deleted proteins that localised mainly in the cytoplasm were degraded to the same extent as wild type protein.

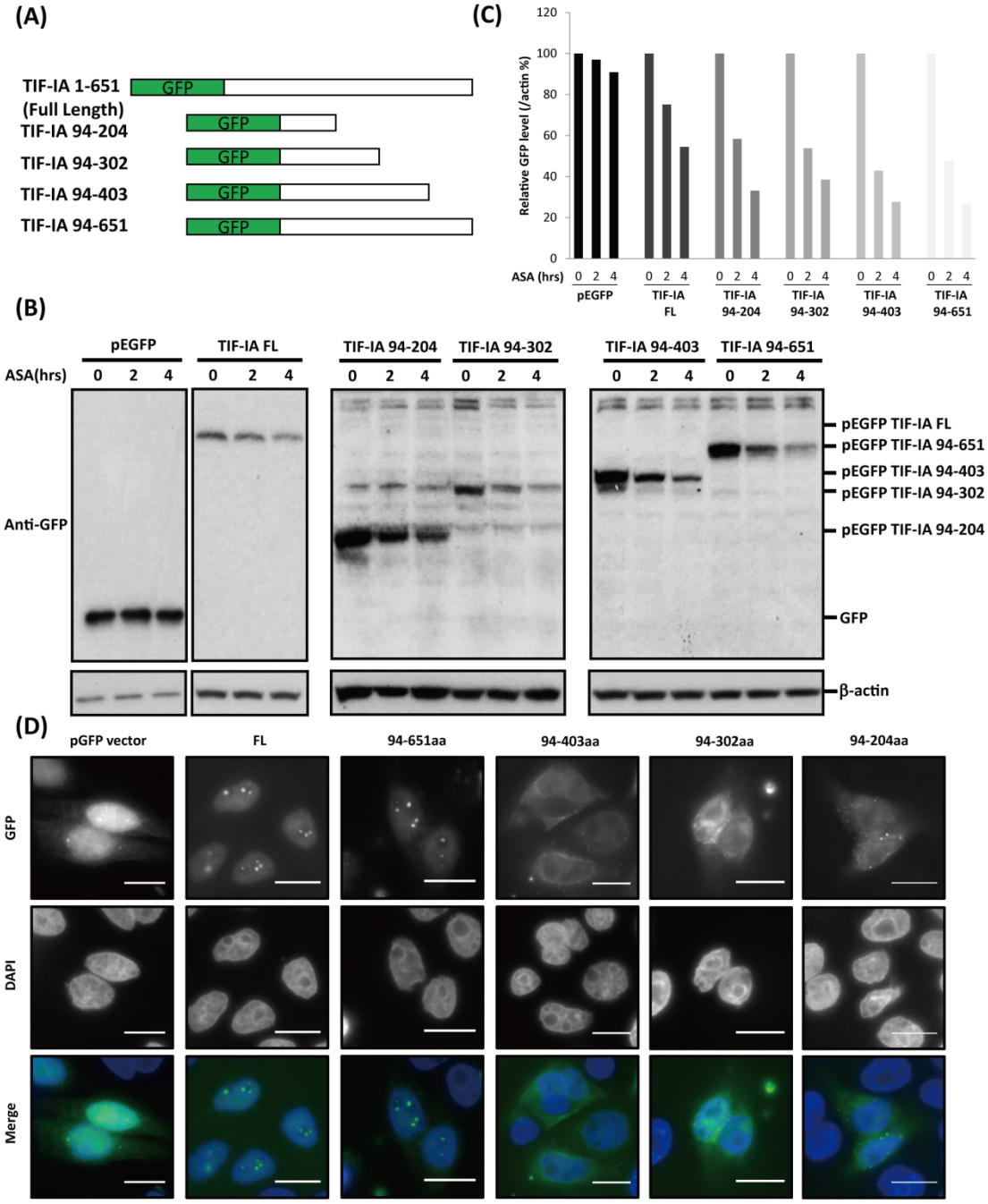


Figure 4.6 94-204 amino acids of TIF-IA are essential for aspirin response.

(A) Domain structure depicting the pEGFP-C1-hTIF-IA deletion plasmids. **(B)** SW480 cells were transfected with the indicated plasmids. pEGFP-C1 vector was used as control to the deletion constructs. 24 hours after transfection, cells were treated with aspirin (10mM) for the indicated times. Whole cells extracts were prepared followed by western blot analysis with anti-GFP antibody. β-actin acted as a loading control. **(C)** Western blot bands from (B) were quantified by Image J. Results shown are relative intensity of GFP normalized by β-actin. **(D)** SW480 cells were transfected with the indicated pEGFP-TIF-IA domain deletion plasmids. pEGFP-C1 plasmids were transfected as control. 24 hours after transfection, cells were fixed with methanol:acetone (1:1) for 30mins before microscope observation. The nuclei DNA is stained by DAPI. All 63X magnification. Scale bars=10um.

4.2.6 The known phosphorylation sites in TIF-IA 94-204aa domain are dispensable for aspirin response

TIF-IA protein has several known phosphorylation sites regulated by mTOR, CK2 and other pathways. Modulation of TIF-IA phosphorylation status at these sites is coupled with changes in cell proliferation and metabolism (Bierhoff et al., 2008; Mayer et al., 2004; Nguyen le and Mitchell, 2013; Zhao et al., 2003). Likewise, analysis based on TIF-IA protein sequence has predicted several potential ubiquitination sites, although no data regarding the bio-function of ubiquitination sites on TIF-IA has been published. A number of the known critical phosphorylation sites of TIF-IA are located between amino acids 94-204. Therefore, I next used site directed mutagenesis to generated mutant pEGFP-C1-hTIF-IA plasmids (Figure 4.7 A), to determine whether any of these sites have a role in aspirin effects on the protein. SW80 cells were transfected with the mutant constructs then anti-TIF-IA western blot analysis used to examine aspirin effects on levels of the mutant proteins. Figure 4.7 B and C show mutating these phosphorylation sites from Serine (S) or Threonine (T) to Alanine (A) (cannot be phosphorylated) or Aspartic (D) acid or Glutamic acid (E)) (mimicking phosphorylation) has no effect on TIF-IA depletion in response to aspirin treatment.

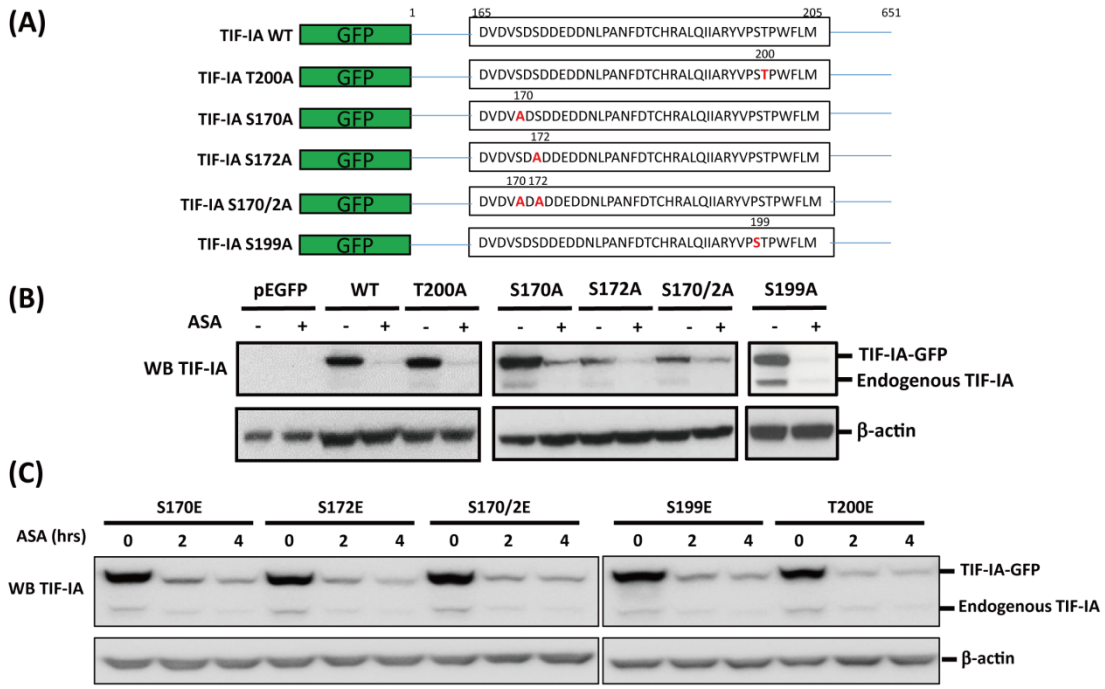


Figure 4.7 Known phosphorylation sites among 94-204aa domain of TIF-IA are not involved in aspirin-induced degradation of this protein.

(A) Site directed mutagenesis was used to generate the phosphorylation site mutants indicated based on the pEGFP-C1-hTIF-IA wild type plasmid. **(B and C)** SW480 cells were transfected with the indicated plasmids or pEGFP-C1 vector as control. 24 hours after transfection, cells were treated with aspirin (10mM) for 4 hours or the indicated times. Whole cells extracts were prepared followed by western blot analysis. TIF-IA and GFP tagged proteins were detected by anti-TIF-IA. β-actin was used as a loading control for all experiments settings.

In agreement with this result, I found modifying known kinase pathways that have been shown to regulate TIF-IA activity through modulation of these phosphorylation sites had a minimal effect on aspirin-induced TIF-IA reduction (Figure 4.8 A).

The mTOR pathway has been shown to regulate serine 44 and serine 199 on TIF-IA (Mayer et al., 2004). In addition, previous studies from this institute and others have shown that aspirin inhibits mTOR activity and thus, activity of the S6 kinase (Din et al., 2012). Therefore, I also further explored this pathway using the inhibitor of mTOR, rapamycin. If mTOR/S6k inhibition plays a role I would expect that rapamycin would mimic the effects of aspirin. Rather I found rapamycin caused an increase in TIF-IA in parallel with decreased phosphorylated S6. Furthermore, this agent had no effect on aspirin-mediated TIF-IA degradation (Figure 4.8 B).

The CK2 pathway targets serine 170 and 172 on TIF-IA (Bierhoff et al., 2008). A previous study demonstrating degradation of TIF-IA had identified a pathway involving CK2-regulated phosphorylation on serine 170 and 172 and MDM2-mediated proteasomal degradation (Nguyen le and Mitchell, 2013). To further explore the role of this pathway in aspirin effects on TIF-IA, firstly, I utilised siRNA to two subunits of CK2, CK2α and β. I found that transfection with the siRNA had no effect on aspirin-mediated TIF-IA degradation (Figure 4.8 C). It did cause a reduction in basal levels of NF-κB as previously reported suggesting the siRNA did cause protein knockdown (Figure 4.8 D).

To further examine the role of this previously published pathway I utilised the MDM2 inhibitor nutlin-3, which stabilises p53 and reportedly blocks MDM2-mediated degradation of TIF-IA (Nguyen le and Mitchell, 2013). I found, although nutlin-3 treatment caused an accumulation of basal TIF-IA protein level and stabilised p53, it had minimal effects on the reduction of TIF-IA protein level in respone to aspirin (Figure 4.8 E). To confirm this process is p53-independent, I further applied HCT116 cells in which the p53 gene has been deleted (Bunz et al., 1998). Figure 4.8 F shows aspirin induces similar degradation of TIF-IA both in parental and p53 deleted (p53-/-) cells. Therefore, these data suggest aspirin mediated TIF-IA depletion is not through the known CK2-MDM2 pathway and is irrespective of p53 status.

Taken together, above data suggested, although the N-terminal 94-204 amino acids of TIF-IA was sufficient for aspirin-mediated TIF-IA dedation, known regulatory phosphorylation sites within this domain were dispensable for this effect.

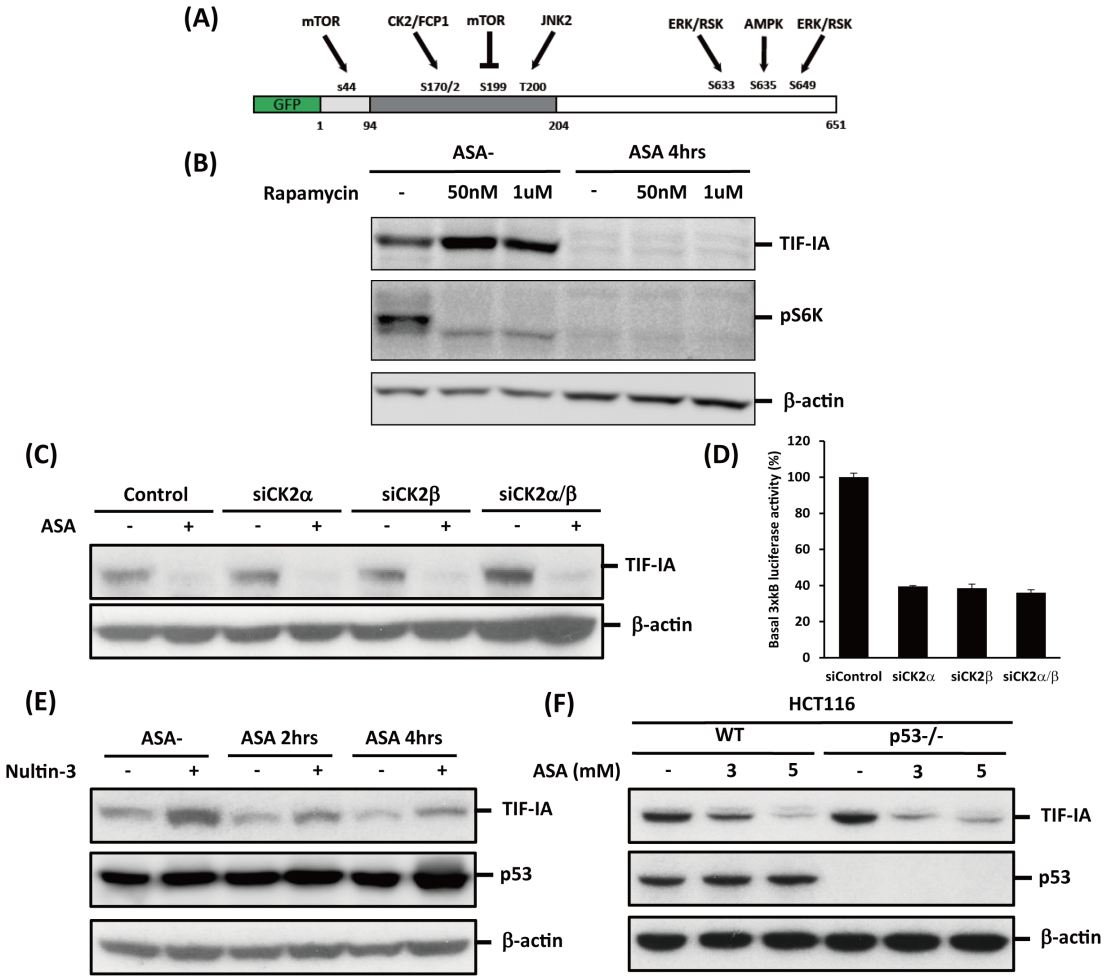


Figure 4.8 Known upstream pathways/kinases are not responsible for aspirin effects on TIF-IA protein reduction.

(A) Schematic showing activating/inhibiting pathways/kinases of TIF-IA and phosphorylation sites known to regulate its activity in response to environmental stress. **(B)** SW480 cells were exposed to rapamycin 50nM or 1uM for 2 hours followed by aspirin (10mM) treatment for 4 hours. Whole cell extracts with phosphoSTOP were prepared and western blot analysis performed to detect TIF-IA and pS6K. **(C and D)** SW480 cells were transfected with indicated siRNA for CK2 along with 3 ug 3x κB ConA-Luc and 1.5 ug pCMV-β. 48 hours after transfection, cells were exposed to aspirin 10mM for 4 hours or non-treated. In (C), whole cell extracts were prepared and western blot analysis performed to detect TIF-IA. β-actin was used throughout to control for protein loading. In (D), NF-κB activity was determined by relative luciferase activity, results were normalized by β-galactosidase activity and are presented as the percentage relative luciferase activity compared to siControl (+/-SD). N=3 technical replicates. **(E)** SW480 cells were pre-treated with Nutlin-3 (5uM) for 24 hours prior to aspirin treatment (10mM for 2 hours or 4 hours). Whole cell extracts was prepared followed by western blot analysis with the indicated antibodies. **(F)** HCT116 wild type or HCT116 p53-/- cells were exposed to aspirin 3mM or 5mM for 24 hours. Whole cell extracts were prepared for western blot analysis. β-actin was used throughout to control for protein loading.

4.2.7 CDK4 lies upstream of stress effects on nucleolus

To further elucidate the mechanism of TIF-IA depletion under stress stimuli, I next examined the upstream signalling involved in this process.

Based on previous studies from the lab and published data showing CDK4 targets the Pol I complex (See 4.1 introduction), I next determined whether CDK4 inhibition lies upstream of stress effects on TIF-IA.

I utilised a chemical CDK4 inhibitor (CDK4i) to mimic stress effects on CyclinD1/CDK4 and found that it caused degradation of TIF-IA (Figure 4.9 A). I also confirmed this effect by using a second CDK4 inhibitor (Figure 4.9 B) and siRNA to CDK4 (Figure 4.9 C). I also found in the time frame that TIF-IA shown dramatic decrease in response to aspirin, CDK4i had no effect on protein levels of CDK4 while inducing degradation of cyclin D1 (Figure 4.9 D).

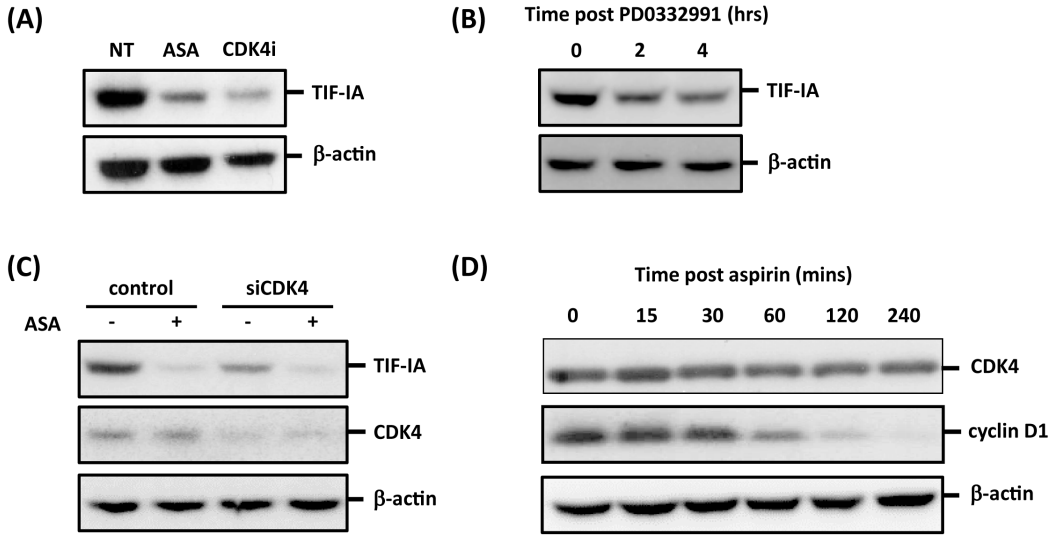


Figure 4.9 CDK4 inhibition mimics aspirin effects on TIF-IA degradation.

(A) Aspirin 10mM or CDK4i 4uM were added to cell culture medium for 4 hours before harvesting whole cell extracts for western blot analysis. Anti-TIF-IA was used to detect TIF-IA protein level. **(B)** SW480 cells were exposed to CDK4 inhibitor PD0332991 (2uM) for the indicated times. TIF-IA protein level in whole cell extract was detected by western blotting. **(C)** SW480 cells were transfected with siRNA against CDK4. 48 hours after transfection, 10mM aspirin was added to medium for 4 hours. Whole cell extracts were prepared and western blot analysis performed to detect TIF-IA and CDK4 protein levels. **(D)** SW480 cells were exposed to aspirin (10mM) for the indicated times then whole cell extracts were harvested. Western blot analysis was performed to analyse CDK4 and cyclin D1 protein levels. β-actin was used as a loading control for all experiments settings.

In addition, the 94-204 amino acids domain of TIF-IA, which is sufficient for aspirin-mediated degradation of this protein, is also important for degradation induced by CDK4 inhibition (Figure 4.10 A), implying that they mediate these effects through a common mechanism. Interestingly, although aspirin-induced TIF-

IA depletion can only be blocked by a combination treatment of proteasome and lysosome inhibitors, CDK4i mediated effect was abrogated by either proteasome inhibitor MG132, lysosome inhibitor Quinacrine or combination of both (Figure 4.10 B to D). Furthermore, similar to aspirin treatment, MDM2 inhibitor nutlin-3 had minimal effects on the reduction of TIF-IA protein level in respone to CDK4i (Figure 4.10 E).

These data, along with previous data from the lab demonstrating that CDK4i mimicks aspirin's effects on nucleolar morphology and rDNA transcription inhibition, strongly suggest CDK4 lies upstream and is involved in aspirin-mediated modulation of the nucleolus.

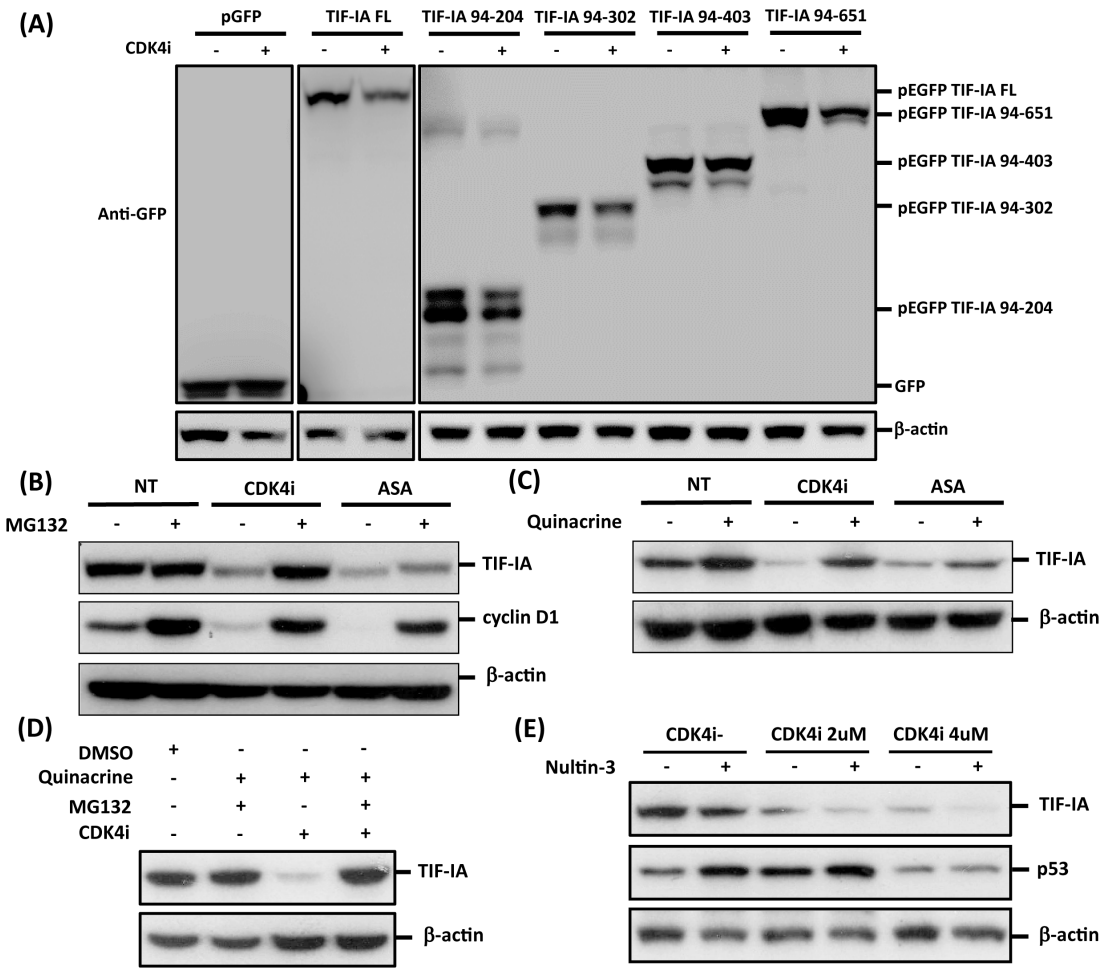


Figure 4.10 CDK4 inhibition utilizes similar mechanism as aspirin to induce TIF-IA degradation.

(A) p-EGFP-TIF-IA domain deletion plasmids (as described in Figure 4.6) were transfected into SW480 cells. CDK4 inhibitor (4uM) was treated for 4 hours after 24 hours of transfection. Whole cell extracts were prepared for western blotting analysis using Anti-GFP antibody. **(B and C)** SW480 cells were pre-treated with MG132 (50uM) for 1 hour (B) or quinacrine (25uM) for 2 hour (C) in prior to aspirin (10mM, 4 hours) treatment. Western blot analysis was performed on whole cell lysates to determine TIF-IA and Cyclin D1 protein levels. **(D)** SW480 cells were pre-treated with both quinacrine (25uM) and MG132 (25uM) for 2 hours prior to aspirin (10mM, 4 hours) treatment. DMSO acts as vehicle control. Anti-TIF-IA western blot analysis was performed on cytoplasmic extracts. α-tublin was used as loading control. **(E)** SW480 cells were pre-treated with Nutlin-3 (5uM) for 24 hours prior to CDK4i treatment (2uM or 4uM for 2 hours). Whole cell extracts was prepared followed by western blot analysis with the indicated antibodies.

4.3 Discussion

In this chapter, I demonstrated for the first time that specific stress stimuli induce the degradation of TIF-IA. I also demonstrate this degradation can take place via a proteasome or lysosomal pathway. An N-terminal 110 amino acid domain (94-204aa) on TIF-IA was found to be sufficient for aspirin-mediated TIF-IA depletion, but the

specific modification sites involved are yet to be identified. I demonstrated that a reported pathway for TIF-IA degradation involving MDM2 is not involved in stress-effects on the protein. Furthermore, I demonstrated that is was independent of p53. CDK4 inhibition mimicked aspirin's effects on TIF-IA suggesting this kinase lies upstream of TIF-IA in response to aspirin.

Stress stimuli induce a reduction in TIF-IA protein levels

Stress that affect rDNA transcription and ribosome biogenesis act predominately by modulating the post-translational modifications on Pol I factors and subsequently altering their activities (Drygin et al., 2010). Pol I factors were shown overexpressed in tumour cells (Negi and Brown, 2015), but studies on the regulation of Pol I factors abundance in response to stress are not profuse. Here, I report stress stimuli of NF-κB induced TIF-IA degradation.

Aspirin was chosen as a model stress inducer in my study, as we are interested in the anti-tumour mechanism of this agent, and the preliminary data in the host laboratory have shown it causes nucleolar morphological changes preceding stimulation of NF-κB pathway. And importantly, in the absence of additional cytokines it stimulates NF-κB in a manner characteristic of multiple stress stimuli (Thoms et al., 2007b). The dose of aspirin used is a critical criterion in epidemiological studies of this agent, therefore this also needs to be considered in *in vitro* studies. The pharmacological related plasma concentration of salicylic acid, the primary hydrolysis product of aspirin, is <3mM (Dovizio et al., 2013). I chose to use 10m aspirin in time course studies investigating the dynamic change of TIF-IA protein level. Firstly, I was convinced I was measuring the direct and primary, but not indirect and secondary, effects of this agent. Secondly, the effects of short time periods treatment with 10mM aspirin on TIF-IA depletion and cellular localisation have also been observed in the long time periods treatment with low dose aspirin (1-5mM).

Beside aspirin, I also showed other stress stimuli of NF-κB induced similar reduction on TIF-IA protein level, including UV-C and ceramide isoforms (Figure 4.2). UV-C stress is a well-studied NF-κB stimulus (Wu and Miyamoto, 2007). Previous results in the lab have shown UV-C exposure caused the similar nucleolar structure change

as aspirin, that is increased nucleolar size and decreased nucleoli numbers. Ceramide is a critical lipid second messenger that integrates stress-activated cell apoptosis through inducing signalling systems including NF-κB (Kimura et al., 2003). Fundamental work in the lab also indicated enlargement/segregation of nucleoli upon treating ceramide isoforms. In contrast, chemical Pol I inhibitors, which had no effect on NF-κB driven transcriptional activity, did not change the protein level of TIF-IA (Figure 4.2). These findings, together with my observation of TIF-IA depletion in response to certain stress inducers, suggest regulation on TIF-IA is probably a common response to stress stimuli of NF-κB. However, I observed treating cells with hydro-peroxide (H_2O_2) and camptothecin (CPT), which stimulated NF-κB pathway (Wu and Miyamoto, 2007) and induced γH2AX foci (done by Lesley Stark), did not induce a decrease in TIF-IA protein level but slightly increased it (Figure 4.2). One consideration is H_2O_2 and CPT have been shown cause different forms of nucleolar stress from aspirin and UV-C. Al-Baker et.al evidenced H_2O_2 did not cause the loss of nucleolar integrity or nucleolar segregation (Al-Baker et al., 2004), and Kalita et.al showed CPT cause nucleolar disruption particularly through inducing the disappearance of NPM in the nucleolus in neuros (Kalita et al., 2008). None of these studies have shown H_2O_2 or CPT cause nucleolar enlargement or nucleoli number changes as we found under aspirin stress or UV-C exposure. Therefore, depletion of TIF-IA may be specifically coupled with a distinct nucleolar stress. I also can not completely rule out the possibility that H_2O_2 or CPT effects on TIF-IA are time- and dose- dependent. Further considerations in this setup would be use different concentrations of H_2O_2 and CPT and perform time-course experiments.

TIF-IA cellular location under stress

Previous studies in the host laboratory have revealed aspirin increased nucleolar size and decreased nucleoli numbers. They also demonstrated aspirin induced a distinct ‘nucleolar cap’ compared to actinomycin D, that is ‘in response to aspirin the FCs and DFC did not completely segregate while there was complete segregation in response to Act D’. Using immunocytochemistry, I found in un-treated SW480 cells TIF-IA formed foci in the nucleolus. Upon treating aspirin, foci were disrupted and TIF-IA became disperse in the nucleolus which was coupled with significant

decrease in nucleolar TIF-IA fluorescence intensity. Although there was no clear 'TIF-IA nucleolar cap' observed in response to aspirin, I found these 'smashed' foci tend to be nucleolar periphery located (Figure 4.1). It was reported TIF-IA was a highly dynamic nucleolar factor that shuttles between nucleolus, nucleoplasm and cytoplasm and TIF-IA translocated out of nucleolus in certain condition (Mayer et al., 2005; Mayer et al., 2004; Szymanski et al., 2009). However, my data was not clear enough to conclude whether TIF-IA translocated out of nucleolus in response to aspirin, with a few of observations supporting an increase of TIF-IA cytoplasmic location, while most of the pictures showed TIF-IA foci dispersed and intensity decrease but still predominately nucleolar location. Indeed, there are some direct supports for the notion that aspirin encourages TIF-IA cytoplasmic translocation before degradation. One is my data on domain deletions of TIF-IA. In these experiments, I found shorter domain deleted proteins (94-204aa, 94-302aa, 94-403aa), which still degraded in response to aspirin, were primarily located in cytoplasm (Figure 4.6). Another one is the previous observations made by the lab members revealed CDK4i, which mimicked most of aspirin effects on nucleolus, induced cytoplasmic accumulation of TIF-IA in prior to degradation of this protein. Hence, I postulate aspirin does cause TIF-IA to translocate out of nucleolus but my images don't capture this process. One explanation for this is from the imaging technical aspect. As reported by Szymański et.al regarding subcellular localisation of TIF, in static status, nucleolus appeared as the brightest cellular structure with TIF-IA and quantifications from single confocal slices showed nucleolus had 23-fold higher concentration of TIF-IA foci than cytoplasmic. However, after measuring compartment volume using confocal z-stack, the nucleolus only obtained 7% of total cellular TIF-IA compared to 48% in cytoplasm and 45% in nucleoplasm (Szymanski et al., 2009). Therefore, it would be with great interest to utilize 3D confocal microscope to further address the dynamics of TIF-IA in response to aspirin.

Aspirin-mediated TIF-IA degradation involves proteasomal and lysosomal degradation pathway

In order to further establish the casual link between stress-induced TIF-IA depletion and stress-induced rDNA transcription inhibition and NF-κB activation, the

mechanism underlying TIF-IA depletion needs to be elucidated. I examined many possible mechanisms to explain this degradation. I demonstrated this depletion is neither a consequence of *TIF-IA* gene transcriptional inhibition nor inhibition of TIF-IA protein synthesis. I found the half-life of TIF-IA protein in SW480 cells is longer than 8 hours, which is contrast with a previous report showing the half-life of TIF-IA is less than 4 hours in U2OS cells (Fatyol and Grummt, 2008). However, considering the different effect arose from different laboratories environment, a more robust comparison would be treating U2OS cells with the same dose of cyclohexamide and to detect the TIF-IA protein level in the same time duration as did in SW480 cells. Moreover, although the cell confluence may influence Pol I transcription and differ the drug treatment outcome (Hannan et al., 2000), I found the reduction of TIF-IA in response to aspirin treatment was not affected by cell confluence either low concentration or over-confluent (Supplementary Figure 4.1 A). Previous data from our lab and others indicates ubiquitin proteasome system proteins increase in abundance in nucleoli in response to aspirin and proteasome treatment and become trapped in nucleolar aggresome containing ubiquitinated proteins. I addressed the possibility that TIF-IA was undetectable due to entrapment in insoluble complexes by using a buffer that can solve aggresomes and different lysis buffers with different detergent concentration followed by western blot analysis (Supplementary Figure 4.1 B and C). Results ruled out the observed TIF-IA reduction in whole cell extract was due to TIF-IA becoming undetectable upon formation of insoluble aggresome in response to aspirin. Furthermore, I also exclude the invovlment of caspase dependent cleavage in this depletion (Supplementary Figure 4.1 D).

Observations in the study have pointed the mechanism underlying aspirin-induced reduction of TIF-IA protein level to protein degradation. Firstly, cycloheximide treatment in SW480 cells indicates the half-life of TIF-IA in this cell is more than 8 hours, while aspirin induced TIF-IA depletion generally happens 30 minutes to 1 hour after treatmen, thus implying protein degradation pathways have been triggered upon the exposure to this agent. Secondly, although separately using proteasome inhibitor and lysosome inhibitor had minor effects on aspirin mediated TIF-IA reduction, inhibiting proteasomal and lysosomal activity together completely blocked

the observed TIF-IA degradation, again suggesting the involvement of protein degradation pathways in this regard.

Previous studies have demonstrated that endogenous TIF-IA is targeted by proteasome dependent degradation, and Sug-1 and MDM2 have previously been identified as TIF-IA E3 ligase (Fatyol and Grummt, 2008; Nguyen le and Mitchell, 2013). However, I found proteasome inhibitors and MDM2 inhibitor nultin-3 had no or minimal effects on preventing aspirin-induced TIF-IA degradation. Besides, fundamental works in the lab demonstrated aspirin did not change the interaction between Sug-1 and TIF-IA, together with my finding that TIF-IA depletion mutant lacking reported Sug-1 binding domain (1-94aa) did not affect aspirin-induced degradation (Figure 4.6), indicating the reported TIF-IA E3 ligase Sug-1 was also not involved in this stress-mediated TIF-IA regulation. Therefore, I postulate it is an unknown E3 ligase involved in stress-mediated TIF-IA modification that selectively target the protein for proteasomal or lysosomal degradation. Moreover, further experiments investigating the ubiquitination of TIF-IA upon aspirin treatment would be desirable.

My study is for the first time to find TIF-IA protein level is regulated by lysosome degradation pathway. I also evidenced although separately using proteasomal or lysosomal inhibitor has minor effects on stress mediated TIF-IA degradation, simultaneously applying these two types of inhibitors can completely abrogate this effect. This indicates a possibility that TIF-IA could be targeted by either of the degradation pathway under stress. This hypothesis could be supported by recent findings showing cooperation between proteasome and lysosome degradation pathways in cells in case of protein overloading and degradation pathways redundancy. In addition, there are some proteins already known to be substrates of both degradation pathways (Lilienbaum, 2013).

The known phosphorylation sites in TIF-IA 94-204aa domain are dispensiable for aspirin response

A set of studies has elucidated the mechanisms underlying stress-mediated TIF-IA inactivation and degradation are through modulation of its phosphorylation status,

and a number of phosphorylation sites and responsive upstream kinase have been identified (Jin and Zhou, 2016). Unlike these known regulations on TIF-IA, I found the stress-stimuli of NF-κB effects on TIF-IA is independent of these known regulatory cascades, by applying TIF-IA domain deletion constructs, mutating known phosphorylation sites and specifically inhibiting upstream regulative kinase pathways.

Firstly, I applied a set of TIF-IA domain deletion plasmids. In the past decades, studies have gradually illustrated the molecular basis of TIF-IA in the regulation of RNA polymerase I initiation and cell growth, that characterised domains on this protein responsible for certain functions (Figure 4.11). For examples, TIF-IA acts as a bridge between Pol I and SL-1 through interacting with two Pol I subunits RPA43 and PAF67 with its C-terminal 512-609aa whereas associating with SL-1 components TAFI95 and TAFI68 on a conserved short motif (411-415aa) (Yuan et al., 2002). DNA binding of TIF-IA is essential for rDNA transcription, and it has been identified TIF-IA binds to rDNA on its 382-400aa (Stepanchick et al., 2013). TIF-IA binds Pol I as a monomer but forms dimer in solution, the 335-493aa on this protein is required for the dimer interface contact (Blattner et al., 2011). 1-94aa on the C-terminus is targeted by E3 ligase Sug-1 for proteasome degradation (Fatyol and Grummt, 2008). In my study, I demonstrated 94-204aa of TIF-IA are sufficient for aspirin response and 403-651aa of TIF-IA are responsible for nucleolar localisation of this protein (Figure 4.6).

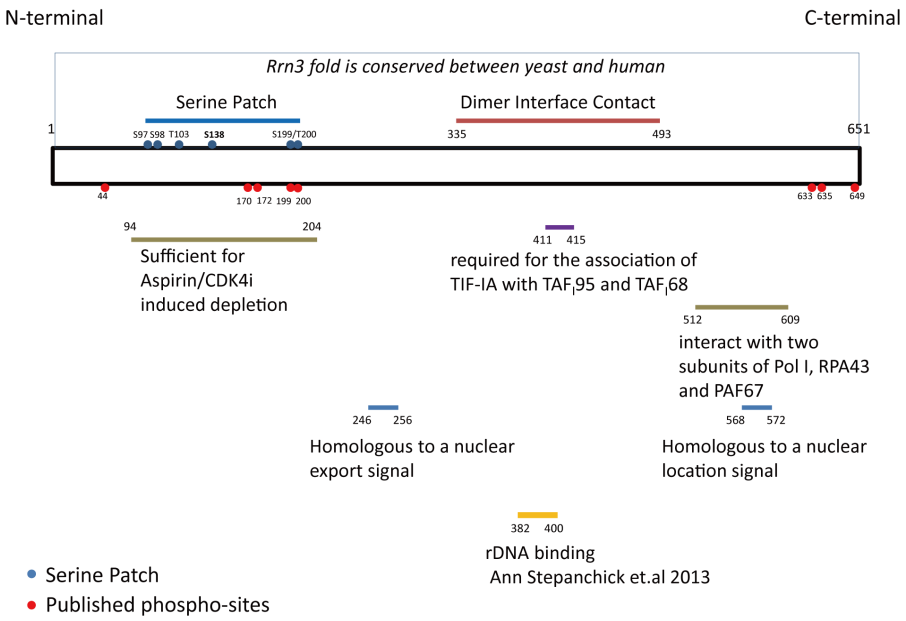


Figure 4.11 Schematic shows previously identified functional domains in TIF-IA.

The 94-204 amino acids domain on TIF-IA is with particular interest, as a structure–function analysis of TIF-IA has revealed a surface serine patch on this protein, which ranges from 97-200 amino acids. In vitro and in vivo functional analysis further demonstrated this serine patch is critically important in Pol I components recruitment, Pol I binding and cell growth (Blattner et al., 2011). In supporting with this finding, most of the previously identified TIF-IA phosphorylation sites, which are under stress stimuli regulation, locate in this domain. To study the involvement of these published phosphorylation sites in aspirin-mediated TIF-IA regulation, I accordingly mutated these sites and transfected into SW480 cells, but none of these mutants blocked aspirin's effects. However, to further strength this data, repeat this transfection in TIF-IA-null cells would be diserable to exclude the effects of endogeneous TIF-IA binding to the mutants. Furthermore, using inhibitors or siRNA silencing, I and previous data in the lab have proved inhibiting the activity of kinases (mTOR, CK2, JNK, AKT) that regulates these known phosphorylation sites during this domain has no effect on aspirin-induced TIF-IA degradation. These data would suggest aspirin modulates TIF-IA on a residue that never been identified.

Another possibility is aspirin's effects on TIF-IA is a combined effect from modulations on multiple sites rather than on a single site. This hypothesis parallels

with the finding that mTOR regulates the activity of TIF-IA is a conjunct effect from modifications on two phosphorylation sites serine 44 and serine 199 in the opposite way (Mayer et al., 2004). Generation of shorter domain deletion mutants based on 94-204aa domain would be also help to this question.

CDK4i lies upstream of stress effects on nucleoli

Previous publication in the host laboratory have demonstrated that aspirin caused degradation of cyclin D1/inhibition of CDK4 through upstream activation of p38, and also evidenced this pathway contributes to the nucleolar translocation of NF-κB/RelA and pro-apoptotic effect of aspirin (Thoms et al., 2007b). The CDK4/cyclin D1 complex has been observed modulating the phosphorylation of Pol I factor UBF and affects rDNA transcription (Voit et al., 1999). Therefore, I determined whether CDK4 inhibition lie upstream of aspirin effects on nucleolus. Interestingly, I found CDK4i mimicked aspirin-induced TIF-IA depletion by using siRNA against CDK4 or chemical CDK4 inhibitors, such as palbociclib, a CDK4/6 inhibitor approved by FDA to be used for breast cancer treatment (Sherr et al., 2016). I also found there was almost complete overlap in the mechanisms between CDK4i- and aspirin-mediated TIF-IA degradation (Figure 4.10) i.e. 94-204aa on TIF-IA are essential, is dependent on both proteasomal and lysosomal degradation pathways and was inhibited in the presence of calyculin A (Figure 6.2D), but not nutlin-3. These results are in line with the previous observations in the lab that CDK4i mimicked aspirin effects on nucleolar morphological change and rDNA transcription inhibition. Taken together, these findings suggest CDK4 inhibition lies upstream of TIF-IA depletion and nucleolar stress in response to aspirin, and also reinforce the possibility that stress regulation on TIF-IA in prior to effects on nucleolar structure change and rDNA transcription inhibition.

Based on the previous findings in the lab, it was postulated p38 activation dependent CDK4i inhibition also the upstream signalling cascade that regulates nucleolar stress response. However, fundamental works in the lab revealed that inhibiting p38 activity, either using chemical inhibitor or siRNA interfere, did not block aspirin's effects on TIF-IA protein levels and rDNA transcription, despite preventing cyclin

D1 degradation in the presence of this agent. This suggests the effects of aspirin on TIF-IA and nucleolar functions are independent of p38 activation-mediated degradation of cyclin D1/CDK4 inhibition. Hence, there are two possibilities: First, an alternative pathway rather than p38 is utilized by aspirin to inhibit CDK4 in prior to affect nucleolus. For this possibility, p16Ink4A is a great candidate to be investigated. p16Ink4A is an inhibitor of CDK4 that through binding to this kinase and inhibiting the interaction between this kinase and cyclin D1 thus inhibiting the catalytic activity of this complex (Serrano et al., 1993). Interestingly, a cohort study has observed the loss of p16Ink4A expression in numbers of colorectal cancers through methylation on promoter of this gene (Shima et al., 2011). Therefore, it is possible that aspirin induces inhibition of CDK4 through upregulating expression of this protein. Indeed, I performed few experiments to examine the p16Ink4A protein levels in response to aspirin. However, although this protein level seems upregulated in 15 minutes of aspirin treatment, which parallels aspirin induced TIF-IA depletion in 30 minutes, this protein appears to be undetectable by whole cell extract based western blot analysis in colon cancer cell lines compared to other cell types (Supplementary Figure 4.2). It would be great interesting to address this question further. Experiments could be designed by using exogenously overexpressing p16Ink4A in colon cancer cells or testing the methylation status of this protein in response to aspirin. Second, another possibility is that CDK4 is not directly regulated by aspirin, instead, CDK4i and aspirin treatment both affect TIF-IA and nucleolar function through converging on a common pathway downstream of CDK4/cyclin D1. In this regard, it would be good to know whether aspirin treatment directly affect the CDK4 kinase activity through operating ELISA. CDK4 mutants that are either constitutively active or inactive could also be applied to distinguishes these two possibilities. In addition, retinoblastoma protein (pRb), which is phosphorylated by CDK4/cyclin D1 in cell cycle progression, is a possible candidate as a common target by aspirin and CDK4 inhibition. It has been also shown accumulation of pRb in nucleolus inhibited the Pol I transcription through binding and inactivating UBF (Cavanaugh et al., 1995). And hyperphosphorylated pRb could be recruited by nucleolar protein NPM and translocates to nucleolus (Takemura et al., 2002). Hence,

it would be interesting to known whether aspirin promotes the phosphorylation of this protein.

It also need to be noted that, from my observation, aspirin-induced TIF-IA degradation could only be blocked by combination of proteasomal and lysosomal inhibitors, whereas using either of them efficiently blocked this degradation in the presence of CDK4i. This would suggest aspirin has more complicated regulations on TIF-IA compared with CDK4i. It is possible that there are other upstream factors involved in aspirin's effects on nucleolus in addition to CDK4.

Aspirin induced TIF-IA degradation is p53-independent

p53 and related pathways are central downstream regulator of nucleolar stress, but as far as I know, whether p53 is involved in the upstream of nucleolar stress in response to stresses is unknown. In my experiment settings using SW480 cells (with three p53 copies but carrying two point mutations R273H and P309S and with constitutively activates p21 (Rochette et al., 2005)), MDM2-p53 interaction inhibitor nutlin-3 and HCT116 cells that have mutants with a disabled TP53 gene (HCT116 p53-/-), I proved aspirin's effects on TIF-IA depletion is p53 independent. Besides, the previous findings in the lab have shown p53 is dispensable for aspirin effects on NF-κB pathway that lies downstream of nucleolar disruption (Chapter 3), and the subsequent induction of cellular apoptosis (Din et al., 2004). Therefore, I believe, in this context, nucleolar stress response and downstream consequences is in a p53-independent way. Notably, a study demonstrated a potent CDKs inhibitor RGB-286638 induced cell death in p53-null multiple myeloma cells, indicated the exsit of p53-independent mechanisms underlying the anti-tumour effect of CDKs inhibitors (Cirstea et al., 2013).

Taken together, data in this chapter strongly suggest TIF-IA degradation is a parallel stress effect with rDNA transcription inhibition and nucleolar structure changes, and provide further insight to understand the molecular link between stress signalling, nucleolar stress and NF-κB activation.

Chapter 5: Results—p14ARF-UBF axis mediates aspirin-induced TIF-IA degradation and colon cancer cell apoptosis

5.1 Introduction

Having found distinct disruption of Pol I complex activates NF-κB signalling (Chapter 3) and stress-stimuli of NF-κB induce rDNA transcription suppression in line with reduction of Pol I factor TIF-IA protein levels (Chapter 4), in this chapter, I set out to find molecular clues about the nucleolar factors involved in stress-induced TIF-IA depletion and rDNA transcription inhibition, in order to demonstrate a functional relationship between stress-mediated effects on TIF-IA and NF-κB activation.

In order to fully understand the anti-tumour mechanism of aspirin, a related study in the host laboratory involving stable isotope labelling by amino acids in Cell Culture (SILAC) study plus sub-compartmental fraction analysis characterised aspirin effects on the nucleolar proteome (by Ian T. Lobb, unpublished data). Compared to conventional western blotting and image analysis, SILAC is a more powerful tool to answer the biological questions about nucleolar stress. Notably, a number of studies from the Lamond lab have established that the nucleolar proteome is complex and dynamic by using this technology (reviewed in section 1.2).

Using SILAC based quantitative proteomics, the host laboratory investigated aspirin effects on the nucleolar proteome. They identified 749 nucleolar proteins of which 21 were significantly increased (>1.6 fold change at 6 hours and 10 hours aspirin treatment relative to 0 hour control) while only five proteins demonstrated a significant decrease in abundance in response to aspirin. The p14ARF tumour suppressor, a critical nucleolar modulator of MDM2/p53, is among these proteins.

p14ARF is a tumoursuppressor that is a critical regulator of the MDM2/p53 pathway. It is generally held in the nucleolus due to interactions with nucleolar proteins such as nucleophosmin (NPM) and MDM2 (James et al., 2014). The interaction between p14ARF and NPM mainly controls the activity and stability of p14ARF, in that NPM prevents ubiquitin-mediated degradation of p14ARF in non-stressed cells (Chen et al., 2010). The formation of a stable complex between p14ARF and MDM2 in the nucleolus contributes to its tumour suppressor properties. p14ARF prevents MDM2

from binding to p53, thus stabilising p53 and initiating p53-mediated cell cycle arrest and apoptosis (Pomerantz et al., 1998).

Although acting as a key activator of p53 signaling pathway under nucleolar stress, studies also shown p14ARF plays a more intricate role in response to stress and in tumour surveillance, and could act independently of the p53 pathway. For example, in order to elucidate the mechanism of p14ARF-regulated nucleolar function in response to stress, a proteomic study had been carried out using wild type or p14ARF-/- mouse embryonic fibroblasts and revealed a RNA helicase-DDX5 (also known as p68) as a p53-independent p14ARF target in ribosomal biosynthesis. They demonstrated, upon p14ARF depletion, DDX5 was enriched in nucleoli, increasingly associated with UBF and bound to rDNA promoter thus promoting rDNA transcription (Saporita et al., 2011).

p14ARF has also been shown to play a direct role in regulating rDNA transcription and ribosome biogenesis. For example, Sugimoto et.al demonstrated induction of p19ARF in mouse NIH 3T3 fibroblasts retarded the processing of rRNA (Sugimoto et al., 2003). In the same system, another study found that besides the aforementioned NPM, p14ARF interacts with another 20 ribosomal biogenesis related proteins, suggesting a negative regulatory role for ARF in ribosomal biosynthesis regulation independent on MDM2 and p53 (Bertwistle et al., 2004). p14ARF was also shown to directly associate with 60 S preribosomal particles and regulated cell growth (Rizos et al., 2006). Moreover, there is direct evidence demonstrating p14ARF negatively regulates rDNA transcription by binding to the rDNA promoter and interacting with Pol I transcription factors. For example, UBF was identified as a p14ARF binding partner and was shown to be hypophosphorylated by overexpression of p14ARF, which caused inhibition of rDNA transcription (Ayrault et al., 2006). Additionally, RNA polymerase I transcription termination factor TTF-I shuttles between nucleoplasm and nucleolus. p14ARF binds to its nucleolar localization sequence thus inhibiting its nucleolar import causing it to accumulate in the nucleoplasm (Lessard et al., 2010).

These data indicate a p53-independent role of p14ARF in the regulation of cell growth and cell death, which is at least in part through suppression of ribosomal

DNA transcription and synthesis. It also underlines the importance of identification of novel p14ARF-binding proteins in understanding the p53 independent functions of p14ARF and nucleolar stress response.

p14ARF has also been shown be involved in NF-κB regulation. Studies by Rocha et.al have revealed that upon oncogenic stress, p14ARF inhibits the NF-κB pathway to prevent cell transformation irrespective of MDM2 and p53 status. It does this by inducing an association between NF-κB RelA and the histone deacetylase HDAC1, which results in repression of RelA-dependent gene expression (Rocha et al., 2003). In addition, they found p14ARF induced the serine/threonine-protein kinase ATR and its downstream kinase Chk1 mediated phosphorylation of RelA at the transactivation domain, thus repressing the transcriptional activation domain of RelA. (Rocha et al., 2005).

Based on the proteomics studies in the host laboratory that identified p14ARF as a downstream regulator of aspirin, and the proficient evidence of p14ARF's involvement in rDNA transcription, ribosomal biogenesis and NF-κB regulations, I decided to investigate the role of p14ARF in stress effects on TIF-IA, nucleoli and NF-κB pathway.

Firstly, I demonstrated p14ARF is required for aspirin-effects on TIF-IA. Secondly, I found that blocking aspirin- and CDK4i induced TIF-IA reduction by modulation on p14ARF abrogates nucleolar stress, NF-κB stimulation and apoptosis. Furthermore, I identified another Pol I factor UBF was involved in the regulation of TIF-IA by aspirin, postulating a novel nucleolar p14ARF-UBF-TIF-IA complex in responding to stress signalling and mediating stress-activation of NF-κB.

5.2 Results

5.2.1 p14ARF regulates aspirin-mediated degradation of TIF-IA

In order to determine whether p14ARF plays a role in aspirin effects on TIF-IA, I first tested the effects of aspirin on p14ARF protein levels. Consistent with the observation in previous SILAC data which indicated p14ARF is reduced in all cell compartments in response to aspirin, I found the protein level of p14ARF was reduced at lower dose aspirin treatment (3mM) for 24 hours (Figure 5.1 A) in SW480 cells. However, higher dose of aspirin (10mM), which reduced TIF-IA protein level in a rapid manner, had minor effects on p14ARF protein level after 2-4 hours (Figure 5.1 B and C). This suggests aspirin effects on TIF-IA are an earlier event than on p14ARF levels. Next, I determined whether p14ARF could directly interact with TIF-IA. Indeed, co-immunoprecipitation assay revealed that p14ARF interactes with TIF-IA in SW480 cells and demonstrated that this interaction was enhanced by aspirin in a time a dose dependent manner (Figure 5.1 D and E).

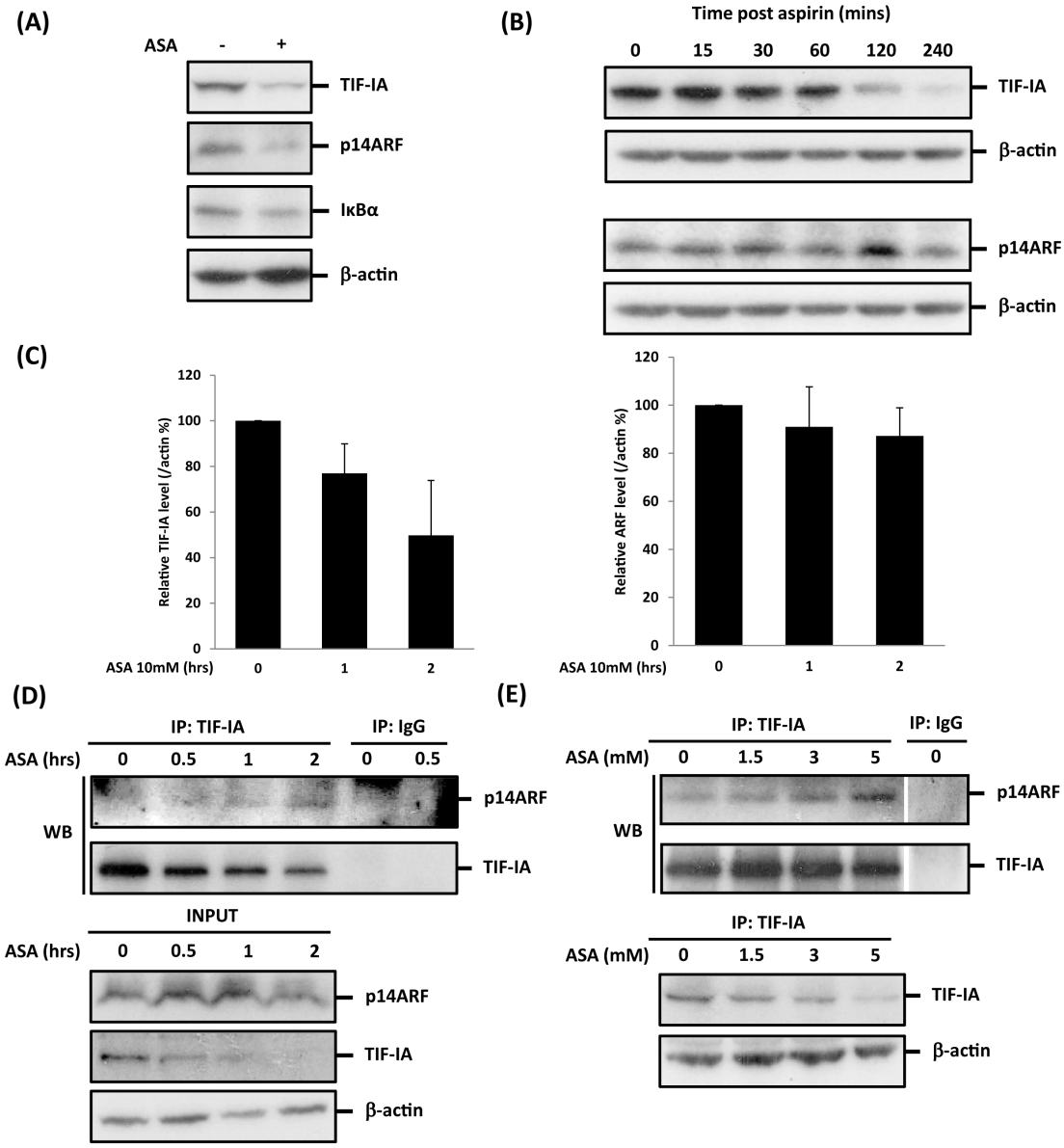


Figure 5.1 Aspirin induces asynchronous reduction of TIF-IA and p14ARF, and promotes the interaction of these proteins.

(A) SW480 cells were exposed to aspirin (3mM 24 hours) or **(B)** aspirin 10mM for the indicated times. Whole cell extracts were prepared for western blot analysis for the indicated proteins. **(C)** Indicated western blot bands from (B) were quantified by Image J. Results shown are relative intensity of TIF-IA or p14ARF normalized by β-actin (+/-SD). N=3 independent experiments. **(D and E)** SW480 cells were treated with 10mM aspirin for the indicated times (D) or aspirin 0-5mM for 24 hours (E). Immunoprecipitation was carried out on whole cell extracts using antibodies to TIF-IA and IgG control. Precipitated proteins were subjected to western blot analysis with the indicated antibodies. Input levels are shown. β-actin was used throughout to control for protein loading.

This interesting result made me further postulate that TIF-IA depletion under aspirin treatment may be directly regulated by p14ARF. To further test this suggestion, siRNA against p14ARF was utilised. SW480 cells were transfected with control or

p14ARF siRNA prior to treatement with aspirin in dose studies. Western blot analysis performed on whole cell extracts indicated that, compared to control siRNA, siRNA depletion of p14ARF attenuated aspirin-induced TIF-IA reduction (Figure 5.2 A and B). Immunocytochemistry analysis confirmed the significant reduction in TIF-IA observed in response to aspirin in cells transfected with control siRNA, was lost in cells transfected with siRNA to p14ARF. Importantly, observations suggested TIF-IA accumulated within nucleoli in p14ARF depleted cells, suggesting a role for p14ARF in nucleolar location or shuttling of this protein (Figure 5.2 C). In keeping with a role for p14ARF, I found that overexpressing the protein enhanced aspirin-induced depletion of TIF-IA (Figure 5.2 D). Together, these data demonstrate p14ARF is involved in aspirin-mediated TIF-IA degradation

p14ARF is located in nucleolus due to its association with nucleophosmin (NPM) (James et al., 2014), it also known that p14ARF regulates rDNA transcription and ribosome biosynthesis through this association (Bertwistle et al., 2004). Therefore, I tested whether the involvement of p14ARF in aspirin-mediated TIF-IA depletion is also through its association with NPM. NPM was depleted from SW480 cells with siRNA interfere, however, this depletion had no effect on TIF-IA depletion or p14ARF reduction upon aspirin treatment, suggesting p14ARF facilitated TIF-IA degradation doesn't require its association with NPM (Figure 5.2 E).

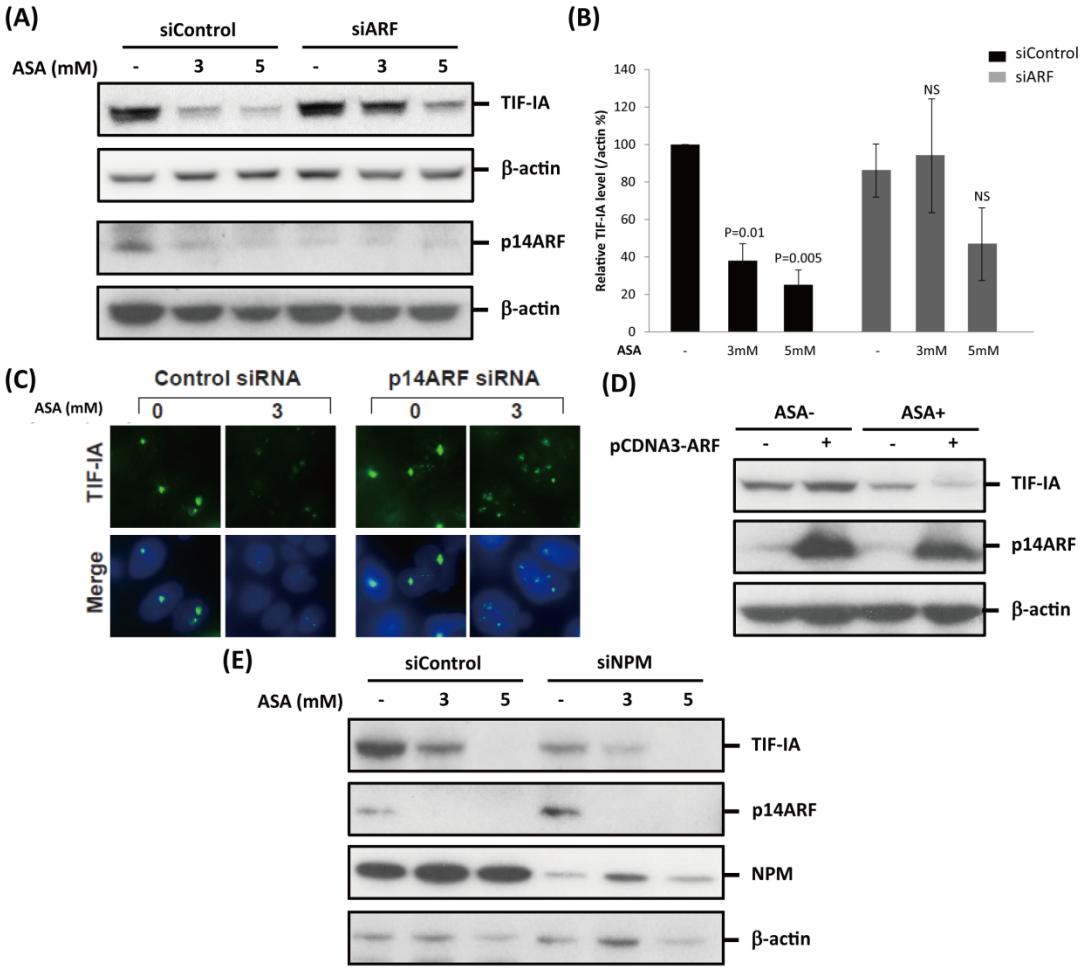


Figure 5.2 p14ARF is a mediator of aspirin effects on TIF-IA depletion.

(A) SW480 cells were transfected with siRNA against p14ARF or a scrambled sequence as control. 48 hours after transfection, 3-5mM aspirin was treated for 24 hours. Whole cell extracts were prepared for western blotting. **(B)** Western blot bands from (A) were quantified by Image J. Results shown are relative intensity of TIF-IA normalized by β-actin (+/-SD). N=2 independent experiments. Two-tailed student's T-test was applied for statistical analysis. p value shows the difference compare to non-treatment control. **(C)** SW480 cells were transfected with control or p14ARF siRNA then treated with aspirin 0-3mM for 16 hours. Immunomicrographs (X63) (pictures taken by Dr. Lesley Stark) show the levels and localisation of TIF-IA in fixed cells. **(D)** SW480 cells were transfected with pcDNA-3 (control) or pcDNA3-p14ARF then either non-treated or treated with aspirin 10mM for 4 hours. Immunoblot was performed on whole cell extract with the indicated antibodies. **(E)** siRNA against NPM were transfected into SW480 for 24 hours before aspirin treatment (3mM or 5mM) for 24 hours. Protein levels were analysed by western blotting. .

p14ARF is differentially expressed in different colorectal cancer cell lines (Figure 5.3 A). To further test the importance of p14ARF in regulating aspirin-mediated TIF-IA depletion, I examined aspirin response in four types of colon cancer cell lines: SW480 and HCT116 (high p14ARF expression), HRT18 and RKO (low/absent ARF expression). Cells were exposed to 1.5mM or 3mM aspirin for 24 hours then whole cell extracts prepared for western blot analysis. Results demonstrate that in response

to aspirin treatment, there was a more robust depletion of TIF-IA in p14ARF expressing cell types (SW480 and HCT116) than the ones without p14ARF expression, supporting the hypothesis that p14ARF is involved in aspirin-induced TIF-IA depletion (Figure 5.3 B and C).

Taken together, these lines of data revealed p14ARF interacts with TIF-IA in response to aspirin and is a regulator of TIF-IA depletion induced by this agent.

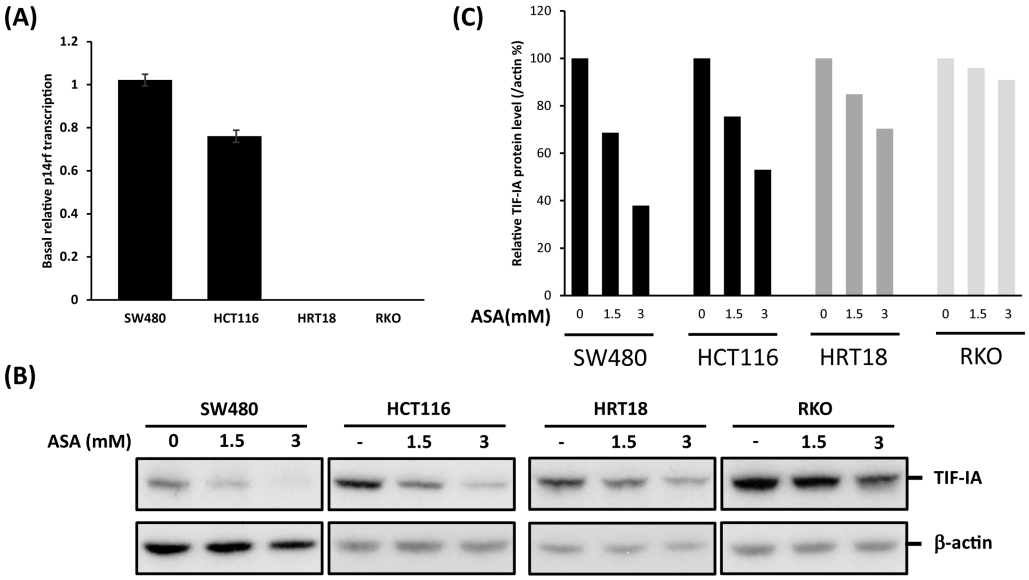


Figure 5.3 Aspirin differentially induces TIF-IA depletion in colorectal cancer cell lines with different p14ARF protein levels.

(A) Total RNAs were extracted from CRC cells subjected to cDNA synthesis. qRT-PCR was performed using Taqman gene expression system. gapdh primer was used as normalization control. SW480 cDNA were used for drawing standard curve. Graph depicts absolute amount of p14ARF mRNA transcripts in different CRCs (+/- SD). N=2 independent experiments. **(B)** CRC cells were exposed to aspirin 0-3mM for 24 hours before WCEs were prepared for western blot analysis. β-actin was used as protein loading control. **(C)** Western blot bands in (B) were quantified by Image J. Results shown are relative intensity of TIF-IA normalized by β-actin.

5.2.2 p14ARF mediated depletion of TIF-IA links nucleolar stress response and NF-κB modulation

Having demonstrated p14ARF is a mediator of TIF-IA depletion in response to aspirin stress, I further studied whether this effect links stress signalling, nucleolar disruption and downstream consequences on the NF-κpathway. Firstly, qRT-PCR analysis revealed siRNA depletion of p14ARF abrogated the repression of rDNA transcription in response to aspirin (Figure 5.4 A and B). In keeping with this finding, immunocytochemistry demonstarted that depletion of p14ARF also blocked the

effects of aspirin on nucleolar morphological changes (Figure 5.4 C and D). These data suggest a causal link between p14ARF-TIF-IA depletion and aspirin-induced nucleolar stress.

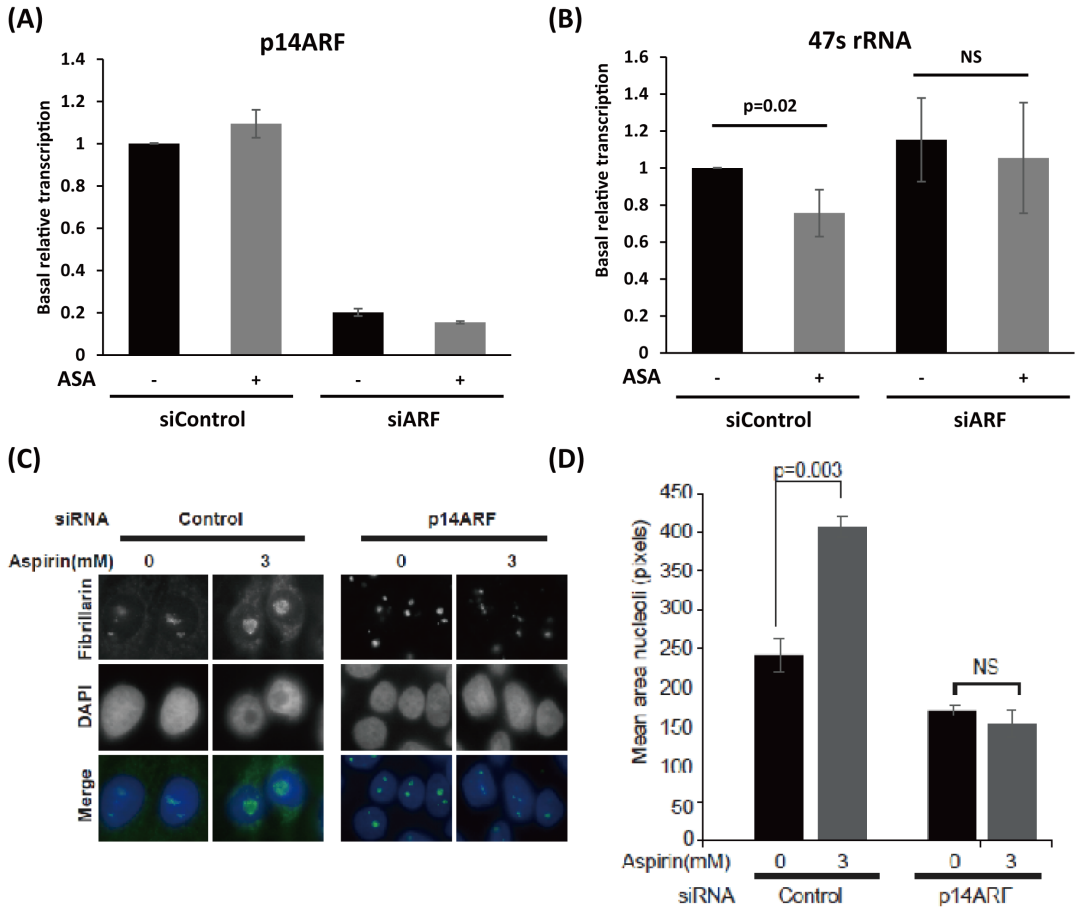


Figure 5.4 siRNA silencing p14ARF blocks aspirin effects on rRNA transcription inhibition and nucleolar enlargement.

(A and B) SW480 cells were transfected with control or p14ARF siRNA for 48 hours before aspirin 3mM treatment for 24 hours. Total RNA were extracted to synthesis cDNA. qRT-PCR were performed by using Taqman-gene expression system with primers set for p14arf and 47s rRNA. gapdh primer was used as normalization control. The graphs depict relative amount of mRNA transcripts comparing to the cells transfected with control siRNA and non-treated (calculated using ddCt algorithm) (+/- SD). N=3 independent experiments. **(C)** SW480 cells were transfected and treated as above. Immunocytochemistry was performed on fixed cells with the indicated antibodies. Fibrillarin (green), DAPI (blue). **(D)** (By Dr. Lesley Stark) Nucleolar area was quantified using IPlab software with fibrillarin as a nucleolar marker. Mean (+/- SEM) of three individual experiments is shown. Two-tailed student's T-test was applied for statistical significance analysis.

Additionally, I found inhibiting TIF-IA depletion using p14ARF siRNA blocked aspirin-mediated activation of the NF-κB pathway, as evidenced by inhibition of IκBα degradation (Figure 5.5 A and B). The effects of p14ARF depletion on TIF-IA

and IκBα were further tested using an independent siRNA to p14ARF, which confirmed that depleting this protein blocks both TIF-IA and IκBα degradation (Figure 5.5 A and B).

Previous data from the lab indicated repression of NF-κB driven-transcription is a downstream consequence of aspirin-mediated IκBα degradation and RelA nucleolar translocation (Stark and Dunlop, 2005). Indeed, 24 hours exposure to 3mM aspirin significantly inhibited NF-κB-driven transcription activity while TNFα (positive control) dramatically enhanced it (Figure 5.5 C). However, knocking down p14ARF significantly attenuated aspirin-induced NF-κB transcriptional inhibition (Figure 5.5 D).

Since previous studies demonstrated p14ARF regulates nuclear NF-κB activity (Rocha et al., 2003) (Rocha et al., 2005), I did consider that depletion of p14ARF could affect stress-mediated NF-κB activation regardless of its regulatory role on TIF-IA. To test this possibility, I examined whether knocking down p14ARF altered the increase in NF-κB activity induced by Pol I complex disruption. Previously, I demonstrated mimicking nucleolar stress by disrupting Pol I complex (siRNA against TIF-IA and UBF) activated the NF-κB pathway (Chapter 3). Here, I found this activation was not changed in the absence of p14ARF, suggesting that in this NF-κB pathway, the role of p14ARF is upstream of Pol I complex disruption (Figure 5.5 E).

Furthermore, in keeping with previous studies showing aspirin-mediated TIF-IA depletion, NF-κB activation and apoptosis are independent of p53, I found that the effects of p14ARF knockdown blocked aspirin effects on TIF-IA and IκBα are independent of p53 (Figure 5.5 F).

Taken together, these findings indicate p14ARF-facilatied TIF-IA degradation is central to sense aspirin or other stresses and convert stress signalling to nucleolar structure modification and downstream NF-κB pathway activation.

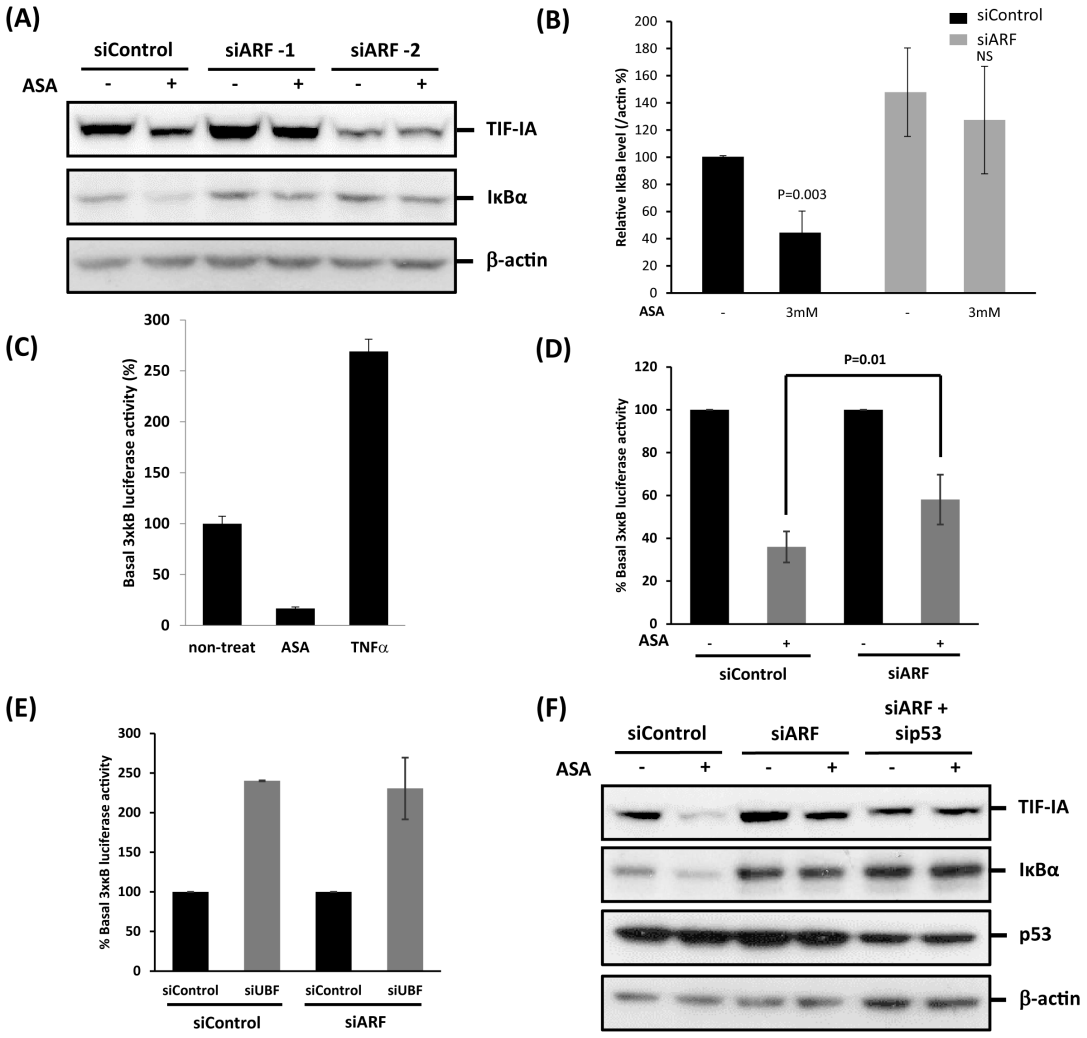


Figure 5.5 p14ARF mediated TIF-IA depletion is causally linked to stimulation of the NF-κB pathway.

SW480 cells were transfected with control or indicated siRNA species for 48 hours. **(A)** Aspirin (3mM 24 hours) was treated. Western blot analysis was performed based on WCE using antibodies against TIF-IA or IκBα. **(B)** Western blot bands from (A) were quantified by Image J. Results shown are relative intensity of IκBα normalized by β-actin (+/-SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical significance analysis. p value shows the difference compare to non-treatment control. **(C to E)** NF-κB activity was determined by relative luciferase activity, results were normalized by β-galactosidase activity and are presented as the percentage relative luciferase activity compared to non-treated samples or siControl (+/-SD). Two-tailed student's T-test was applied for statistical significance analysis.**(C)** 3 ug 3x κB ConA-Luc and 1.5 ug pCMV-β were co-transfected into SW480 cells. 24 hours after transfection, cells were treated with aspirin (3mM for 24 hours) or TNFα (10ng/ml for 4 hours). N=3 technical replicates. **(D)** Luciferase plasmids above were co-transfected along with indicated siRNA. Cells were non-treated or treated with aspirin (3mM 24 hours). N=4 independent experiments. **(E)** Luciferase plasmids above were co-transfected with indicated siRNA. N=2 independent experiments. **(F)** siRNA transfected cells were treated with aspirin 3mM for 24 hours before western blotting were performed based on WCE.

5.2.3 CDK4 lies upstream of p14ARF-facilitated TIF-IA depletion

Previous results suggested CDK4 kinase inhibition lies upstream of aspirin-mediated degradation of TIF-IA. Therefore, I next examined the role of p14ARF in CDK4i-mediated TIF-IA degradation. In keeping with this suggestion, I found that siRNA depletion of p14ARF in SW480 cells blocked CDK4i-mediated TIF-IA degradation (Figure 5.6 A and B). In further support for a role of TIF-IA degradation in stress-mediated activation of the NF-κB pathway, I found that siRNA to p14ARF blocked CDK4i-mediated nuclear translocation of RelA (Figure 5.6 C).

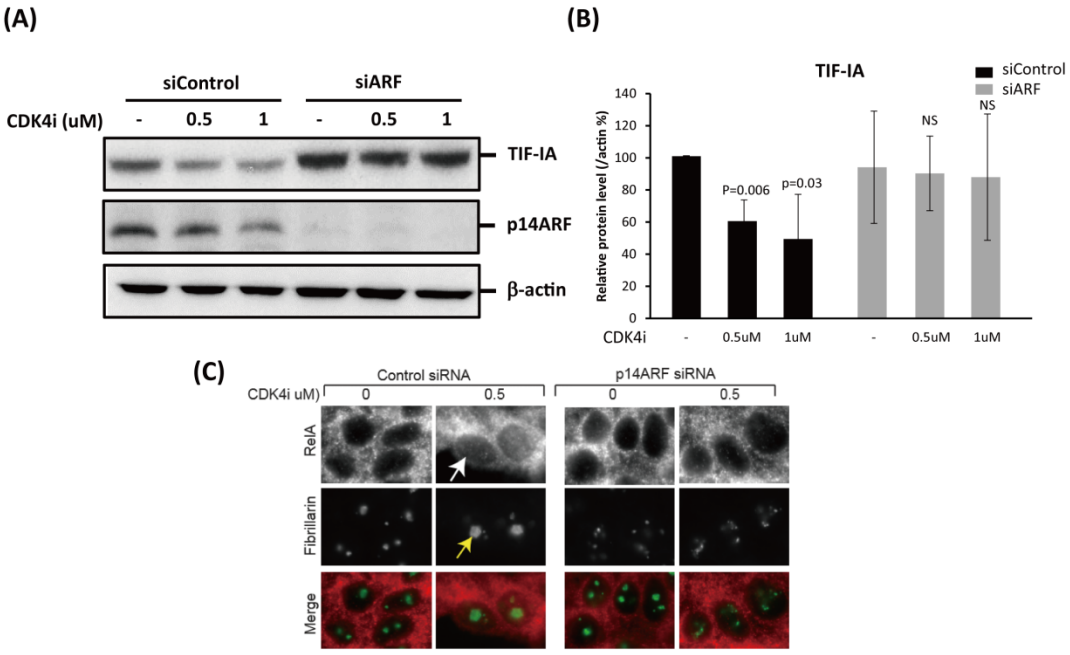


Figure 5.6 p14ARF mediates TIF-IA degradation and NF-κB activation in response to CDK4 inhibition.

(A) Control or p14ARF siRNA were transfected into SW480 cells for 48 hours. Then cells were treated with 0uM, 0.5uM or 1 uM CDK4i for 16 hours. Western blotting was performed on WCE using indicated antibodies. **(B)** Western blot bands from (A) were quantified by Image J. Results shown are relative intensity of TIF-IA normalized by β-actin (+/-SD). N=3 independent experiments. Two-tailed student's T-test was applied for statistical significance analysis. p value shows the difference compare to non-treatment control. **(C)** SW480 cells were transfected with control or p14ARF siRNA then treated with CDK4i for 16 hours. Immunomicrographs (X63) (pictures taken by Dr. Lesley Stark) show the localisation of RelA and fibrillarin in fixed cells.

To further examine the relationship between CDK4i effects on TIF-IA and p14ARF, I examined CDK4i-mediated TIF-IA degradation in cell lines with variable cellular levels of p14ARF expression (Figure 5.7 A). I found that like aspirin, CDK4i-

induced TIF-IA depletion is in accordance with the cellular protein level of p14ARF. For example, compare the effects of CDK4i on TIF-IA in SW480 cells with high levels of p14ARF and U2OS cells with low levels of this protein (Figure 5.7 B and C). In keeping with a connection between TIF-IA degradation and CDK4i-mediated activation of the NF-κB pathway, I found that CDK4i-induced IκBα degradation was attenuated in U2OS cells (Figure 5.7 B and D).

Therefore, in addition to the previous observations that CDK4 acts as an upstream mediator of stress-mediated TIF-IA depletion and nucleolar stress, these findings further demonstrate CDK4 induced TIF-IA depletion is p14ARF dependent and precedes activation of NF-κB.

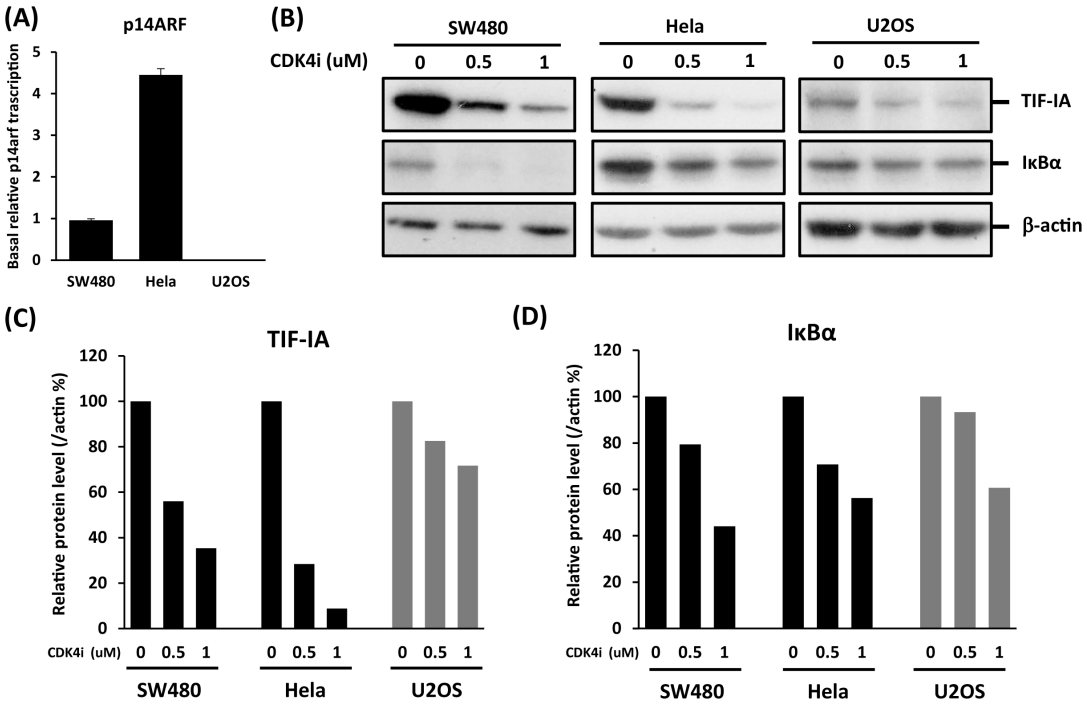


Figure 5.7 Cell lines with different p14ARF protein levels response differentially to CDK4i-induced TIF-IA depletion and IκBα degradation.

(A) Total RNAs were extracted from indicated cells subjected to cDNA synthesis. qRT-PCR was performed using Taqman gene expression system. gapdh primer was used as normalization control. SW480 cDNA were used for drawing standard curve. Graph depicts absolute amount of p14ARF mRNA transcripts in different cell types (+/-SD). N=3 technical replicates. **(B)** Indicated cells were exposed to CDK4i 0-1uM for 16 hours before WCEs were prepared for western blot analysis using anti-TIF-IA and anti-IκBα. β-actin was used as protein loading control. **(C and D)** Western blot bands in (B) were quantified by Image J. Results shown are relative intensity of TIF-IA or IκBα normalized by β-actin.

5.2.4 UBF is involved as an intermediate in p14ARF-dependent TIF-IA degradation

The Pol I transcription complex contains several factors that associate with each other to orchestrate and modulate the activity of rDNA transcription. Of these, UBF is particularly interesting as it is critical for Pol I-driven transcription and is a direct target for both CDK4 and p14ARF. Indeed, CDK4 has been shown to phosphorylate UBF on serine 484, impairment of which halts rDNA transcription and causes cell cycle arrest (Voit et al., 1999).

To determine the role of this protein I firstly examined the phosphorylation status in response to aspirin and CDK4i. I found that depletion of TIF-IA was paralleled by the dephosphorylation of UBF at this known s484 phosphorylation site (Figure 5.8 A), suggesting modification on this site may be required for the aspirin stress response.

To further examine the role of this protein I next used siRNA to UBF. I demonstrated that TIF-IA depletion in response to aspirin and CDK4i is blocked in the absence of UBF, while siRNA silencing of another Pol I subunit, RPA194, had no effect on levels of the protein (Figure 5.8 B and C). This suggests that stress effects on TIF-IA may occur indirectly though modulation of UBF but not Pol I subunit.

The above data indicated a role for UBF in this stress response pathway. To determine whether serine 484 was necessary, we utilised an expression plasmid that expresses a serine to alanine mutation at this site and therefore cannot be phosphorylated. If phosphorylation was important I would have expected that this mutant would mimick the effects of CDK4i/aspirin on TIF-IA levels, or at least enhance the effects of this agent. SW480 cells were transfected with control or UBF siRNA alongside WT UBF or a UBF S484A mutant. Results show that the inhibition of CDK4i-mediated TIF-IA depletion observed in the absence of UBF is rescued by overexpression of wild type UBF. However, expression of the mutant actually blocked CDK4i-mediated TIF-IA degradation. (Figure 5.8 D).

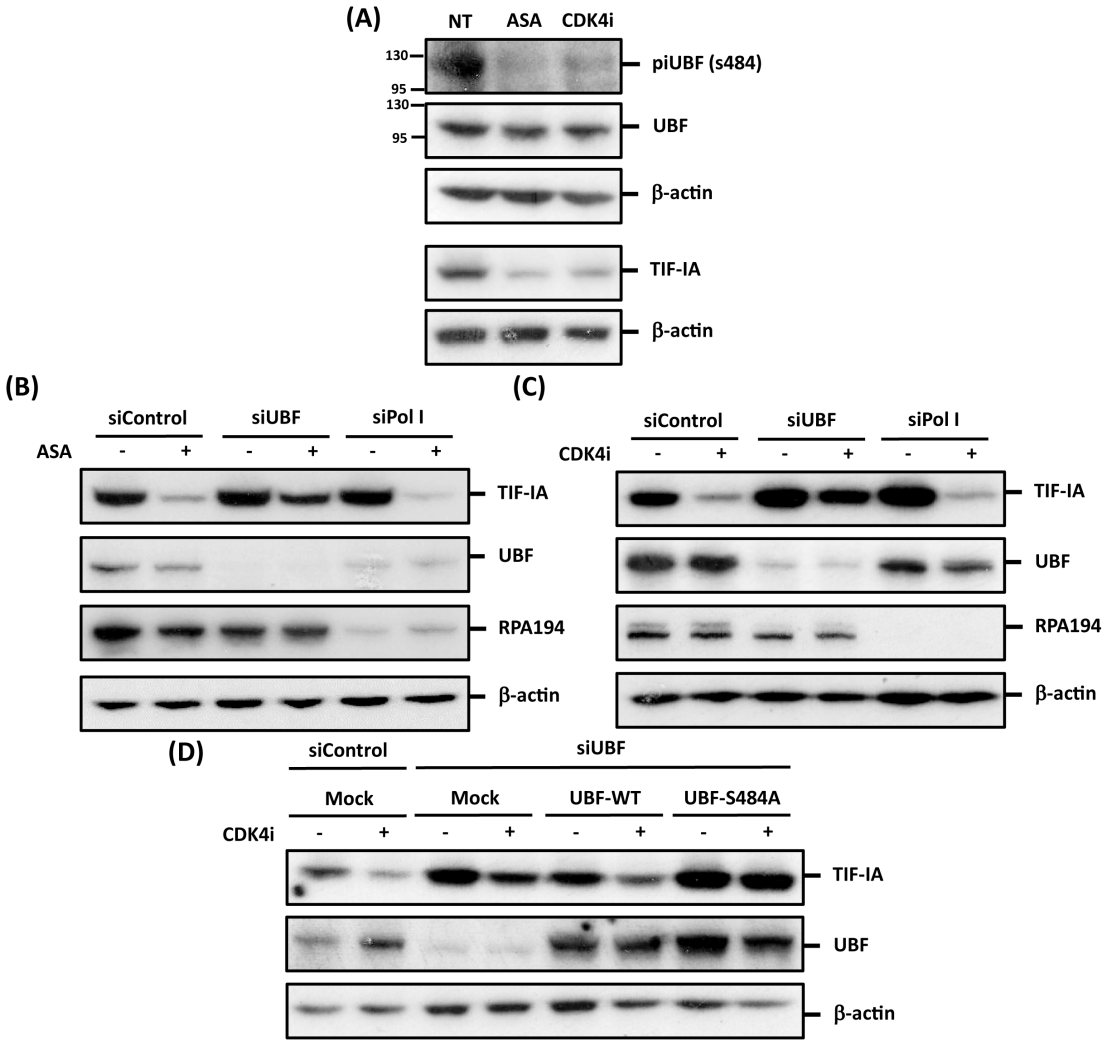


Figure 5.8 UBF is an intermediator of aspirin/CDK4i induced TIF-IA depletion.

(A) SW480 cells were non-treated or treated with aspirin (10mM) or CDK4i (4uM) for 4 hours. WCE were prepared in the presence of phosphoSTOP. Western blot analysis was carried out using indicated antibodies. **(B and C)** SW480 cells were transfected with control, UBF or PolRIA siRNA for 48 hours. Then cells were treated with 10mM aspirin (B) or 4uM CDK4i (C) for 4 hours. Western blottings were performed on WCE with indicated antibodies. **(D)** SW480 cells were transfected with control or UBF siRNA for 48 hours. Then Flag tagged wild type (WT) or site mutated (S484A) UBF plasmids or mock was transfected into cells. 24 hours after plasmids transfection, CDKi (4uM 4 hours) was treated. Western blotting was performed on WCE by the indicated antibodies. β-actin was used throughout to control for protein loading.

To definitively establish a connection between inhibition of CDK4 activity, disruption of the Pol I complex and activation of the NF-κB pathway I next examined whether this UBF-S484A mutant could modulate CDK4i effects on NF-κB activation. SW480 cells were transfected with control or UBF siRNA prior to overexpression of Flag-UBF-WT or -S484A as above. Immunocytochemistry (picture taken by Dr. Lesley Stark) revealed that for both control and UBF siRNA, CDK4i induced a significant increase in nuclear levels of RelA in cells transfected

with wild type UBF, but not in those transfected with the S484A phospho-mutant (Figure 5.9).

Taken together, these results suggest UBF acts as an accessory in aspirin- and CDK4i- mediated TIF-IA depletion and NF-κB activation. They also suggest a role for S484 of UBF although whether phosphorylation at this site is involved remains unclear.

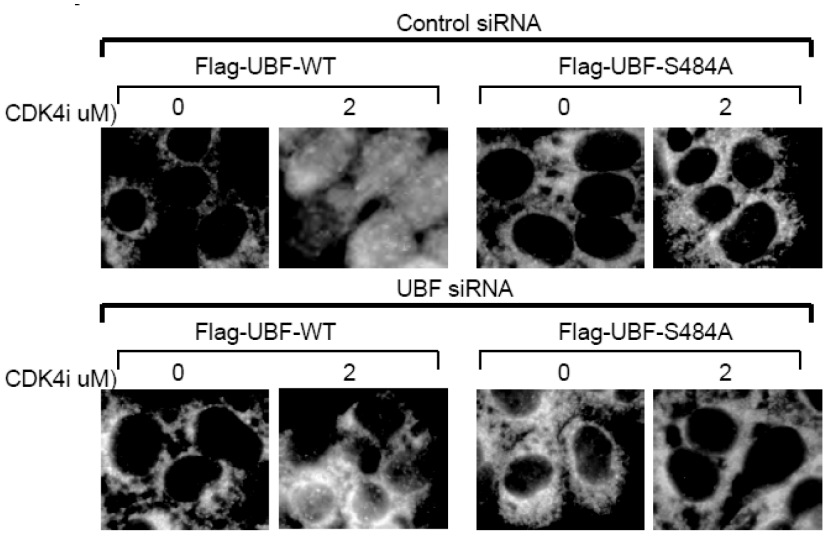


Figure 5.9 UBF S484A mutant blocks CDK4i effects on RelA nuclear translocation.
SW480 cells were transfected with the specified siRNA species then co-transfected with Flag-UBF WT or Flag-UBF S484A expression vectors. Immunomicrograph demonstrates the localisation of RelA after CDK4i (2uM 16 hours) treatment. 63X magnification for all images. (Images taken by Dr. Lesley Stark)

5.2.5 Relationship between p14ARF-facilitated TIF-IA degradation and pro-apoptotic effects of aspirin

Previous studies in this lab have shown that degradation of IκBα is absolutely required for aspirin and CDK4i-mediated apoptosis. To investigate the relationship between the identified nucleolar stress response pathway and pro-apoptotic activity of aspirin, I performed apoptosis assay. In SW480 cells, p14ARF was silenced by siRNA prior to aspirin treatment for 24 hours. Apoptotic cells were then determined by positive Annexin V staining. I found knocking down p14ARF, basally attenuated cell apoptosis. Aspirin treatment significantly enhanced apoptosis in control cells, which was completely inhibited in cells in which p14ARF was silenced (Figure 5.10

A). Whole cell extracts were also prepared from cells in the same experiment setup for western blot analysis, revealing aspirin induced a reduction of pro-caspase 3 level, a hallmark of apoptosis, in paralleled with TIF-IA depletion, and importantly, both of effects were blocked by siRNA silencing p14ARF (Figure 5.10 B). This was further confirmed by an independent p14ARF siRNA, which also suggested this effect was coupled with aspirin effects on NF-κB activation (Figure 5.10 C).

Taken together, these data suggest a potential connection between aspirin-mediated p14ARF-facilitated degradation of TIF-IA, activation of NF-κB pathway and pro-apoptotic effects of this agent.

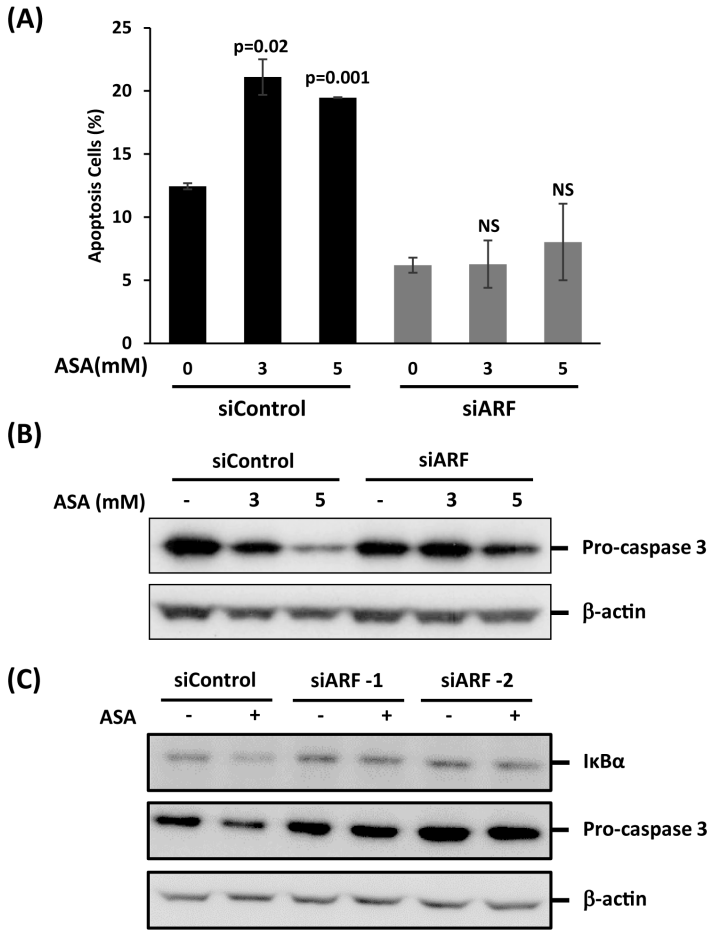


Figure 5.10 siRNA silencing p14ARF blocks aspirin-induced TIF-IA depletion, as well as cell apoptosis and cleavage of pro-caspase 3.

(A) SW480 cells were transfected with p14ARF siRNA or a scrambled sequence for 48 hours prior to aspirin treatment (3mM or 5mM for 24 hours). Apoptosis assay was performed by using Annexin V-FITC apoptosis detection kit. Apoptotic cells with positive Annexin V staining were sorted by FACS, and the percentages of apoptotic cells within the total cell populations were calculated (+/- SD).Graph depicts the apoptotic percentage mean of at least 20,000 total cells. Two-tailed student's T-test was applied for statistical significance analysis. p value shows the difference compare to non-treatment control. N=3 independent experiments. **(B and C)** SW480 cells were transfected with control or two distinct p14ARF siRNA for 48 hours. Then cells were treated with 3-5mM aspirin (B) or 3mM aspirin (C) for 24 hours. Western blottings were performed on WCE with indicated antibodies. β-actin was used as protein loading control.

5.3 Discussion

In this chapter, I elucidated a novel nucleolar stress response pathway that through regulating a CDK4-UBF-p14ARF-TIF-IA axis culminates in the activation of the NF-κB pathway. I demonstrated p14ARF interacts with TIF-IA in response to stress, and is a mediator of aspirin/CDK4i mediated TIF-IA degradation. Blocking TIF-IA

degradation through siRNA silencing of p14ARF abrogated stress effects on rDNA transcription inhibition, nucleolar morphological changes, stimulation of NF-κB and most importantly, the induction of cell apoptosis. I also revealed UBF was involved as an intermediate and serine 484 on UBF was required in this regulation. Taken together, these data proposed a novel nucleolar stress response pathway that lies upstream of NF-κB activation, and this could potentially explain the anti-tumour effects of aspirin in colorectal cancer.

p14ARF plays a role in stress-mediated TIF-IA degradation and rDNA transcription inhibition

Since aspirin treatment down-regulates p14ARF in nucleolus, I initially postulated that reduced p14ARF was linked to TIF-IA depletion. However, contrary to this expectation, western blot analysis revealed siRNA silencing of p14ARF actually abrogated aspirin-mediated degradation of TIF-IA, especially at lower concentration of this agent (Figure 5.2). In contrast, overexpression of p14ARF enhanced TIF-IA degradation in the presence of aspirin (Figure 5.2). This seems counterintuitive, but the experiments on the detailed kinetic studies on TIF-IA and p14ARF protein level and immunoprecipitation indicated aspirin-mediated degradation of TIF-IA precedes loss of p14ARF (Figure 5.1), and these two proteins actually associated upon aspirin exposure. Immunocytochemistry analysis further confirmed p14ARF was a mediator of TIF-IA degradation, and we found although TIF-IA degradation was blocked in the cells transfected with p14ARF siRNA, TIF-IA foci was trend to accumulate at the periphery of nucleoli rather than in the central as found in control cells (Figure 5.2 C). pEGFP-p14ARF plasmids were also employed to study the co-localization of TIF-IA and p14ARF. I found p14ARF was predominately accumulated in nucleolar and nucleoplasm in SW480 cells, but it was not clear to conclude whether p14ARF and TIF-IA co-localised and travelled out of nucleolus in response to aspirin from these results (Data not shown). Further experiments on 3D confocal imaging would help address this question. Taken together, these data would suggest that p14ARF contributes to TIF-IA degradation in response to aspirin possibly through binding and transporting it to the cytoplasm for proteasomal or lysosomal dependent degradation.

The association between p14ARF and nucleolar protein NPM contributes to the nucleolar location of p14ARF and regulation on rDNA transcription and ribosome biosynthesis of this protein (Bertwistle et al., 2004; James et al., 2014). I found this association was not required for p14ARF in mediating TIF-IA degradation in stress condition. However, I did interestingly observe the basal level of TIF-IA was decreased in the presence of siRNA against NPM and aspirin-induced degradation of TIF-IA was slightly enhanced by knocking down NPM. This would suggest an interesting possibility that depletion of NPM actually disrupted its interaction with p14ARF thus releasing p14ARF to bind TIF-IA for degradation. This possibility should be further explored in future experiments.

Taken together, previous studies have suggested p14ARF is a negative regulator of rDNA transcription in a p53/MDM2 independent manner, and a few nucleolar proteins have been identified as p14ARF partners in this regulation. Here I demonstrate another mechanism of p14ARF regulating rDNA transcription under stress conditions is through facilitating TIF-IA degradation.

CDK4i-p14ARF-UBF axis regulates stress effects on TIF-IA

In chapeter 4, I provided data to show that CDK4 inhibition mimics aspirin effects on TIF-IA and NF-κB activation, and discussed the possibility that CDK4 lies upstream of nucleolar stress in response to aspirin. Here, I conclude that the UBF-p14ARF axis lies downstream of CDK4i effects on TIF-IA. This conclusion is supported by a line of observations. First, CDK4i causes p14ARF depletion. Second, siRNA against p14ARF blocked CDK4i-induced TIF-IA degradation and NF-κB activation. Third, CDK4i and aspirin induce decrease of UBF phosphorylation on serine 484. Fourth, knocking down UBF and introducing UBF-S484A mutant abrogated CDK4i-mediated TIF-IA degradation. Taken together, these results would suggest aspirin and CDK4i induce TIF-IA degradation through a common pathway involving UBF-p14ARF-TIF-IA axis. Now it would be imperitive to show that aspirin exert nucleolar stress directly through regulation on CDK4 by demonstrating aspirin has no further effect on TIF-IA/nucleoli and NF-κB signalling in the presence of the CDK4 inhibitor.

RNA polymerase I transcription machinery contains several components that have complicated interactions with each other to coordinate the function of rDNA transcription. Therefore, it is possible that stress-mediated TIF-IA degradation is 'assisted' by TIF-IA's 'teammates'. Among them, I demonstrated UBF is involved. Firstly, I found siRNA silencing UBF in SW480 cells completly blocked aspirin- and CDK4i- induced TIF-IA degradation (Figure 5.8). Secondly, aspirin and CDK4i both down-regulate phosphorylation of UBF at serine 484, and introducing UBF-S484A mutant abrogates TIF-IA degradation in the presence of these agents (Figure 5.8). Thirdly, and importantly, expressing UBF-S484A mutant impaired CDK4i induced NF-κB activation (Figure 5.9). These data suggest the involvement of UBF and requirement of modification on serine 484 of UBF in this regulation.

Having found aspirin and CDK4i cause a reduction of UBF phosphorylation and that siRNA against UBF activates NF-κB to a larger extent than siRNA against TIF-IA (Figure 3.1 and Figure 5.8), it could be arguing that it is stress effects on UBF, rather than TIF-IA, that are critical for downstream stimulation of NF-κB. However, evidence here indicates that loss of p14ARF abrogates both aspirin and CDK4i mediated activation of NF-κB which is coupled with inhibition of TIF-IA degradation. Therefore, I propose TIF-IA degradation is critical in stress response and connecting stress signalling to downstream activation of NF-κB, while disruption of other Pol I components could mimic the consequences of TIF-IA degradation

It will now be of considerable interest to elucidate how UBF is involved in the interaction between p14ARF and TIF-IA and what the role of S484 is. Interestingly, Ayrault et.al revealed one of the nucleolar partner of p14ARF is UBF, and UBF was hypophosphorylated and lost the ability to recruit other Pol I components to rDNA promoter upon overexpressing of p14ARF, suggesting an association between these two proteins in regulating rDNA transcription (Ayrault et al., 2006). Therefore, I postulate that hypophosphorylation of UBF, probably serine 484, or a switch of phosphorylation status at this residue, is required to mediate the interaction between p14ARF and TIF-IA. To test this expectation, further experiments using co-immunoprecipitation to examine the association between p14ARF and TIF-IA under stress in the presence of UBF siRNA or UBF-S484A mutant, or *vice versa* would be

helpful. Based on the observations from Ayrault et.al (Ayrault et al., 2006), another possibility could be that p14ARF actually lies upstream of UBF. That is, stress-enhanced interaction between p14ARF and TIF-IA promotes p14ARF-induced hypophosphorylation of UBF, and this hypophosphorylation impairs the rDNA recruitment of Pol I components including TIF-IA, thus inducing the disassociation of this protein from rDNA and translocation out of nucleolus for degradation. Further experiments designing to test the phosphorylation status of UBF in the presence of siRNA against p14ARF and Chromatin immunoprecipitation (ChIP) assay of Pol I components on rDNA, especially TIF-IA, are desirable to address this hypothesis.

Based on my data, I conclude that UBF-p14ARF axis lies upstream of TIF-IA degradation. I suggest this pathway is only active in response to specific stresses as overexpression of p14ARF or siRNA silencing p14ARF or UBF has minimal effects on TIF-IA basal protein levels. Although my data suggests that p14ARF-facilitated TIF-IA degradation is a mechanism linking stress, NF-κB activation, and aspirin-induced apoptosis, based on my data so far I cannot rule out the possibility that other nucleolar pathways are involved in this process. Especially as I found that in some cell lines with very low levels of p14ARF, aspirin and CDK4i still induce TIF-IA degradation to a reasonable level (although not as significant as the cell lines with p14ARF expression).

TIF-IA degradation is directly linked to stimulation of NF-κB pathway

Previous chapters in the thesis have demonstrated nucleolar disruption activate NF-κB pathway and stress stimuli of NF-κB induce TIF-IA degradation in prior to effects on NF-κB pathway. In this chapter I have provided evidence to prove stress-mediated TIF-IA degradation is directly linked to nucleolar enlargement and stimulation of the NF-κB pathway. For example, siRNA against p14ARF, which blocked stress-mediated TIF-IA degradation, also blocked stress effects on rDNA transcription inhibition, nucleolar enlargement and NF-κB activation. Applying UBF-S484A mutant, that abrogated CDK4i-mediated TIF-IA degradation, also prevented CDK4-induced RelA nuclear accumulation. In addition to these cell line studies, observations made from clinical samples strongly support this notion.

Biopsies of fresh surgically resected human colorectal tumours were exposed to pharmacological dose of aspirin (100uM) for 1 hour. Western blot analysis revealed aspirin induced significant decrease of TIF-IA protein level in 4 out of 7 tumours, meanwhile quantitative immunohistochemistry analysis found significant increase of $pRelA^{s536}$ staining in 3 out of 6 tumours. Importantly, they found there was a strong inverse relationship (r^2=-0.85, n=6) between aspirin-induced TIF-IA protein level changes and the percentage of cells with phosphorylated RelA. These data in the primary human tumours confirm the strong relationship between aspirin-induced TIF-IA degradation and activates NF-κB pathway, and indicate the clinical significance of this nucleolar stress response pathway.

Previous studies have shown nucleolar fragmentation and stabilisation of p53 is a downstream consequence of inactivating TIF-IA through alteration on its phosphorylation status (Mayer and Grummt, 2005). Here, based on the findings mentioned above, the observations that TIF-IA degradation was always associated with nucleolar enlargement and NF-κB activation under certain stress stimuli of NF-κB (aspirin, UV-C, ceramide), and siRNA silencing TIF-IA alone enhanced NF-κB-driven transcriptional activity, I propose the downstream consequences of inactivating TIF-IA through stress-mediated degradation are nucleolar enlargement and NF-κB activation.

Nucleolar stress response pathway containing UBF-p14ARF-TIF-IA axis plays a role in the pro-apoptotic effect of aspirin

There are overwhelming evidence supporting that aspirin is effective in prevention and treatment of cancers, especially colorectal cancer (discussed in Chapter 1). Although numbers of molecular mechanisms have been proposed for the anti-tumour effects of this agent, molecular markers could be efficiently applied for predicting the potential side effects and anti-tumour outcome of this agent are very limited. This study was aimed to make progression on this area, and I did suggest this novel nucleolar stress response pathway containing UBF-p14ARF-TIF-IA axis plays a role in the pro-apoptotic effect of aspirin, through observations on pro-caspase 3 immunoblot analysis and apoptosis assay in the presence of p14ARF siRNA under

aspirin exposure (Figure 5.10). Besides, works done by other lab members in colorectal tumour *ex vivo* model, which indicate a strong inverse relationship between aspirin-mediated TIF-IA degradation and percentage of cells with phosphorylated RelA, have also shown the clinical significance of this pathway. The significance of this nucleolar stress response pathway in the pro-apoptotic effect of aspirin will be further discussed in the final discussion (Chapter 7).

Chapter 6: Results—Other signals regulating TIF-IA in response to aspirin

6.1 Results

6.1.1 Mass spectrometry shows other signals regulate TIF-IA in response to aspirin

Results above suggest the nucleolar response pathway containing UBF-p14ARF-TIF-IA directly links stress signals to NF-κB activation and also induction of cell apoptosis. However, I cannot completely rule out the possibility that other factors or pathways are involved in this process. It would be also interesting to find unknown signals that regulate the relationship between UBF-p14ARF-TIF-IA. Therefore, we next used label-free quantification (MaxLFQ) to investigate TIF-IA interacting proteins under aspirin treatment. This approach is ideal as MaxLFQ intensities not only accurately represent dynamic changes in protein interactions, but can also be used to rank the proteins in terms of likely relative abundance.

I immunoprecipitated endogenous TIF-IA and specifically detected it at high intensity using LC-MS/MS. Generally, SW480 cells were exposed to 10mM aspirin before harvesting and whole cell lysates prepared with NP-40 cell lysis buffer. Antibodies against TIF-IA (or IgG control) were used as baits for potential TIF-IA associated factors (Figure 6.1 A). Immunoprecipitation (IP)-mass spectrometry for protein-protein interactions and initial raw data process using MaxQuant platform were carried out by the Edinburgh Cancer Research Centre (ECRC) facility with support from Jimi Wells (ECRC) and Alex von Kreigsheim (ECRC), who is an expert in this technology (Turriziani et al., 2014). Three independent experiments and quantifications were applied.

From the total interactomes, the lists of potential proteins specifically interacting with TIF-IA in the absence or presence of aspirin (ASA- or ASA+ respectively) were identified based on data analysis using average LFQ intensity ratio and *T*-test *p* value. Basically, proteins having the TIF-IA/IgG ratio of average LFQ intensity (relative LFQ ratio) higher than 2 and *p* value less than 0.05 were considered to specifically interact with TIF-IA. Using this cut-off I identified 26 proteins that specifically interact with TIF-IA in the absence of aspirin and 20 proteins in the presence of aspirin (Figure 6.1 C and E, Appendix Table 8.1 and 8.2).

STRING database, which is a web resource predicting protein-protein interactions mainly based on experimental evidence, computional prediction and textmining of published abstract (Szklarczyk et al., 2017), was applied to visualize the associations between these identified proteins (Figure 6.1 B and D). As expected, the majority of these were ribosomal proteins (including TIF-IA (RRN3)) and subunits of RNA polymerase that have known or predicted interactions with TIF-IA, thus confirming the specificity of the technique. However, I also identified some novel TIF-IA interactors, such as the BRCA1-A complex subunit Abraxas (encoded by *FAM175A* gene) in the absence of aspirin, which would be with great interest for further study (Figure 6.1 B-E, Appendix Table 8.1 an 8.2).

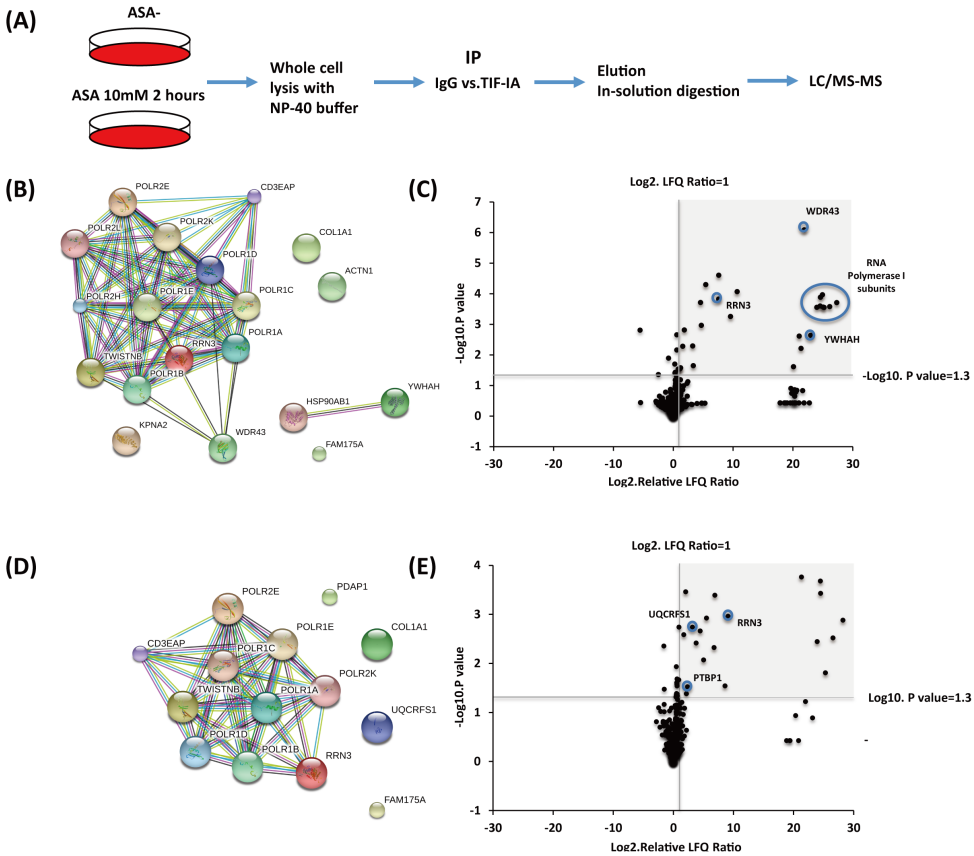


Figure 6.1 Mass spectrometry analysis shows other signals regulating TIF-IA in the absence or presence of aspirin.

(A) Graph describes the protocol of the 'on-beads digestion in conjuction with data-dependent mass spectrometry' analysis. **(B and D)** Candidate proteins specifically interact with TIF-IA in (B) ASA- or (D) ASA+ were identified based on the cut-off (average LFQ intensity ratio >2, t-test p value <0.05). Diagrams from STRING Database show these proteins and their known interactions. **(C and E)** Plots created using relative LFQ ratio (TIF-IA/IgG) measured in three biological replicates. The $\log_2$ of mean ratios were plotted on the x-axis and the $-\log_{10}$ of p value, calculated using a two-sided T-test, were plotted on the y-axis. Candidates having the relative LFQ ratio higher than 2 and p value less than 0.05 were considered to specifically interact with TIF-IA in the absence (C) or in the presence (E) of aspirin, and highlighted with grey background.

To identify factors that are differentially binding to TIF-IA under aspirin treatment, I compared the proteins that specifically bound to TIF-IA in ASA- and ASA+ conditions. There were 17 candidates shared by ASA- and ASA+ lists. While there were 9 proteins specifically interacting with TIF-IA in ASA-, and 3 proteins in ASA+, indicating a dynamic fluctuations of TIF-IA interactors in the presence and absence of aspirin. (Figure 6.2A). Among these proteins specifically interact with TIF-IA in the absence of aspirin, majority of which are subunits of RNA polymerase I, suggesting this agent disrupted the Pol I-TIF-IA interaction and potential TIF-IA-involved complexs (Figure 6.2 A and B). With particular interest, 14-3-3 protein eta (encoded by *YWHAH* gene) and WD repeat-containing protein 43, also known as Utp5 (encoded by *WDR43* gene) were two proteins displaying most significant and consistent decrease of relative LFQ intensity under aspirin treatment (Figure 6.2 B and 6.1C). 14-3-3 protein eta belongs to 14-3-3 proteins family, which involved in the regulation of multiple aspects of signalling pathways through recognizing and binding to phosphoserine or phosphothreonine motif. WDR43 is involved in ribosomal biogenesis and is mainly localized in fibrillar centre of the nucleolus. The proteins specifically interact with TIF-IA under aspirin treatment include Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (encoded by *UQCRFS1* gene), which known to be located in mitochondria and involved in respiratory chain; and Polypyrimidine tract-binding protein 1 (encoded by *PTBP1* gene), which is a nuclear ribonucleoproteins (hnRNPs) and involved in pre-mRNA processing regulation (Figure 6.1 E and 6.2A).

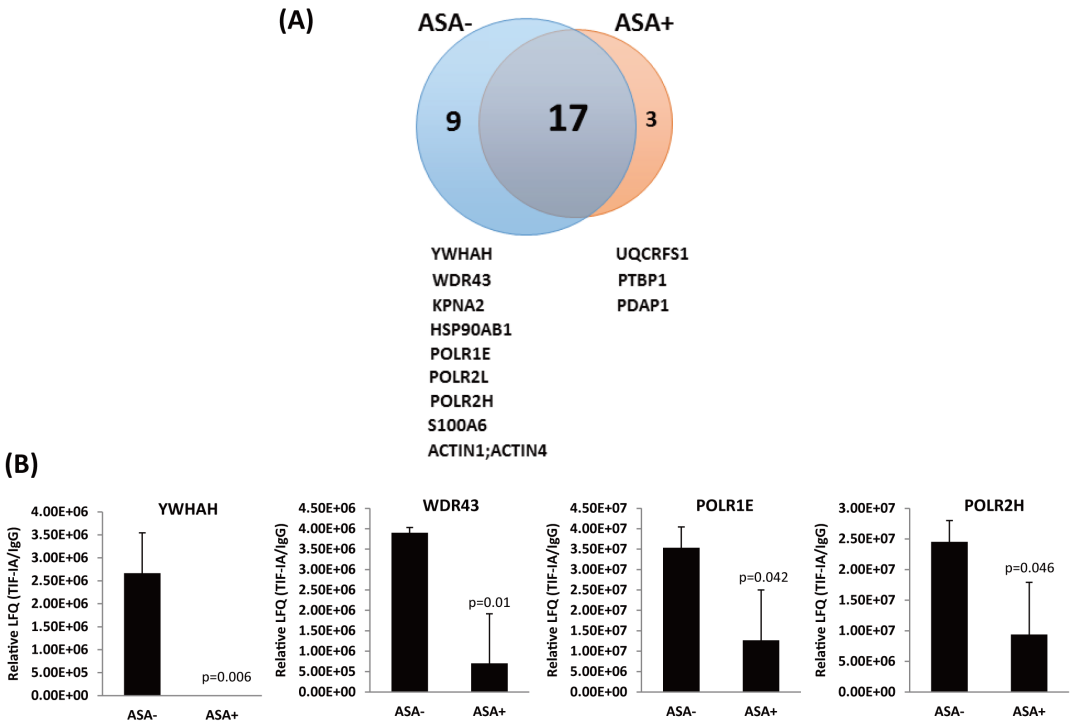


Figure 6.2 Mass spectrometry analysis shows proteins differentially interact with TIF-IA under aspirin treatment.

(A) Venn diagram shows the number of potential proteins that are shared by ASA- and ASA+ or specifically identified in these two conditions. Gene names are listed below. **(B)** Graphs show the relative LFQ intensity (TIF-IA/IgG) (+/-SD) of indicated candidates under ASA- and ASA+ condition. *p* value shows the difference between ASA- and ASA+. N=3 independent experiments.

6.1.2 Mass spectrometry identified phosphorylation on serine 44 of TIF-IA is modulated by aspirin treatment

I have shown that proteasomal degradation (which is dependent on ubiquitination) is required for aspirin-mediated TIF-IA degradation (Figure 4.5). I have also shown this is dependent on p14ARF, which generally influences the activity of interacting proteins through modulation of their sumoylation status. Therefore, I postulate a key role for post-transcriptional modifications (PTMs) in this regulation. Label-free quantification (MaxLFQ) can simultaneously investigate TIF-IA PTMs and interacting proteins. Alex von Kreigsheim (ECRC) has recently used a similar approach to identify substrates of PHD3 and FIH20 (Rodriguez et al., 2016).

Surprisingly, data analysis identified serine 44 on TIF-IA as the only active phosphorylation sites modified by aspirin. Phosphoryaltion at this site was significantly reduced in response to aspirin treatment (Figure 6.2 A). This seems

incompatible with my previous results show 94-204 amino acids of TIF-IA protein are sufficient for aspirin response. To confirm the role of this residue, I applied two previously generated site-mutated TIF-IA plasmids as described in Chapter 4 (4.2.6): a serine to alanine mutation at residue 44 TIF-IA (GFP-TIF-IA S/A) that cannot be phosphorylated and a serine to aspartic acid (GFP-TIF-IA S/D) mutation that mimics phosphorylation. These constructs were transfected into SW480 cells then treated with aspirin in dose response studies. I found that mutating S44 to alanine (A) slightly enhanced aspirin-mediated degradation of TIF-IA while mutating the residue to aspartic acid (D) partially attenuated this effect (Figure 6.2 B). These results suggest that dephosphorylation at this site is important in aspirin-mediated TIF-IA degradation.

To further examine whether de-phosphorylation was involved in aspirin-mediated effects on TIF-IA, I applied general serine/threonine phosphatase inhibitor Calyculin A. I found both high dose (50nM) Calyculin A and low dose (5nM) Calyculin A completely abrogated TIF-IA's reduction under aspirin or CDK4i treatment, suggesting de-phosphorylation process was required for this modulation (Figure 6.2 C and D).

Therefore, these observations suggests that aspirin-mediated de-phosphorylation of TIF-IA is critical for the subsequent degradation of the protein.

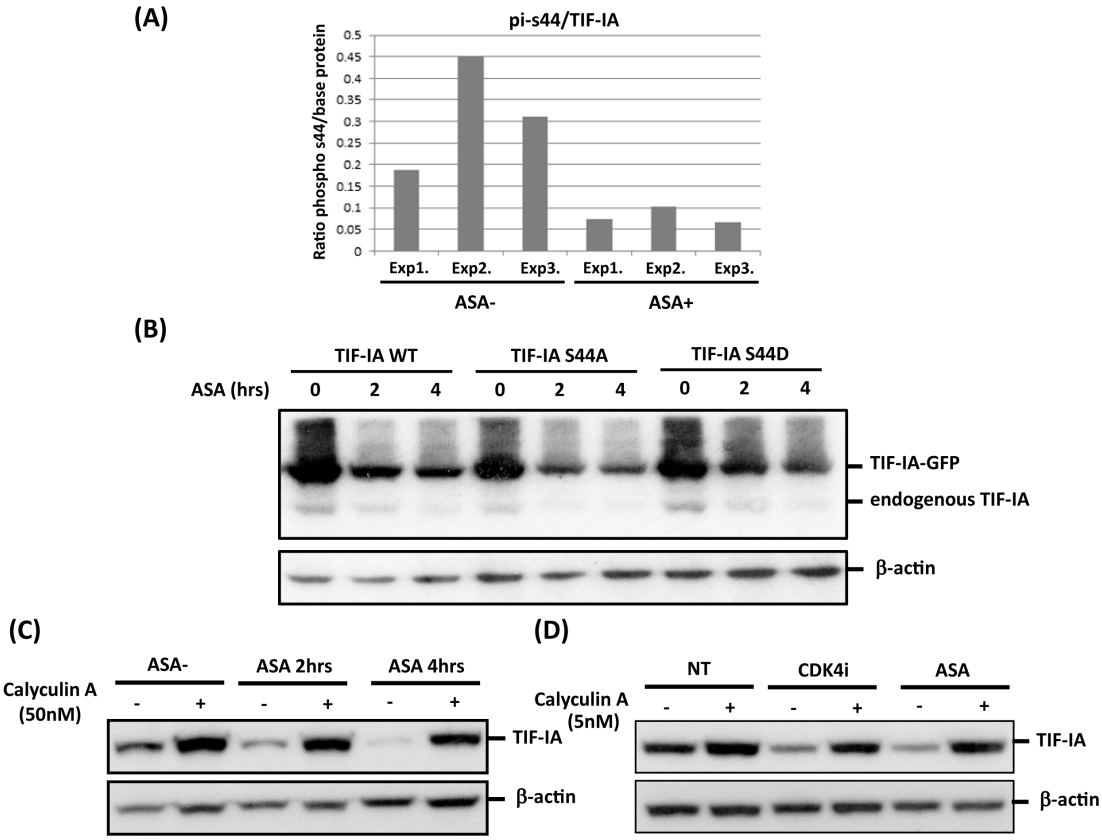


Figure 6.3 Mass spectrometry shows phosphorylation on serine 44 of TIF-IA is down-regulated by aspirin treatment.

(A) After fragmentation in mass spectrometry analysis, graph depicts the level of phosphorylation on serine 44 of TIF-IA/base protein ratio (analysis carried out by Jimi Wills and Alex von Kriegsheim in the MRC Institute of Genetics & Molecular Medicine). **(B)** Site directed mutagenesis was used to generate the phosphorylation site mutants indicated based on the pEGFP-C1-hTIF-IA wild type plasmid. SW480 cells were transfected with the indicated plasmids. 24 hours after transfection, cells were treated with aspirin (10mM) for 4 hours for the indicated times. Whole cells extracts were prepared followed by western blot analysis. TIF-IA and GFP tagged proteins were detected by anti-TIF-IA. β-actin was used as a loading control. **(C and D)** SW480 cells were exposed to Calyculin A 50nM for 2 hours or 5nM for 4 hours followed by aspirin (10mM) treatment or CDK4i (4uM) for 4 hours. Whole cell extracts were prepared and western blot analysis performed to detect TIF-IA.

6.2 Discussion

Although my data suggest that a UBF-p14ARF-TIF-IA axis plays a role in stress-mediated degradation of TIF-IA, there are still a number of questions need to be answered regarding this pathway. For example, the factors mediate Pol I complex disruption and NF-κB activation are still unknown; and the detailed mechanism underlying the interplay between UBF, p14ARF and TIF-IA is unknown. To identify novel proteins and post-translational modifications involved, I ustilised 'on-beads digestion in conjuction with data-dependent mass spectrometry'. These data

suggested a number of novel TIF-IA interactions in response to aspirin. Among them, 14-3-3 protein eta is with most significant changes, and with most interesting due to their known celluar functions. 14-3-3 protein eta is a member of the 14-3-3 proteins family which are highly conserved and widely expressed across eukaryotic organisms. 14-3-3 proteins recognize and bind to the phosphoserine and phosphothreonine motif of their targeted proteins or kinases, thus regulating their kinase activities, cellular location or protein interactions. The role of 14-3-3 proteins in regulating cell cycle progression has been extensively studied. In particular, 14-3-3 protein eta was shown to regulate mitotic progression, and deletion of it resulted in enhanced mitotic cell death and sensitized tumour cells to microtubule inhibitor, suggesting this protein as a potential target for cancer therapy (Lee et al., 2013). 14-3-3 protein eta was also shown to regulate the cell cycle through interacting with CDC25, a dual phosphatase that regulates and activates CDKs (Mils et al., 2000). Furthermore, a study revealed colon cancer cells (HCT116) with a 14-3-3 protein eta deficiency were more sensitive to p14ARF-induced apoptosis, indicating a potential link between 14-3-3 protein eta and p14ARF, which has not been investigated so far (Hemmati et al., 2008).

Besides the well-studied role in cell cycle regulation, 14-3-3 proteins are also involved in the regulation of other signaling pathways, some of them, particularly MDM2 and mTOR, are with great interest regarding this study. Wood NT. et.al have shown 14-3-3 proteins interact with Pim kinase phosphorylated MDM2 (Wood et al., 2009), which implies a potential role of 14-3-3 proteins in mediating protein degradation. Therefore, further experiments in this study investigating whether 14-3-3 protein eta is involved in aspirin-induced TIF-IA depletion will be beneficial. 14-3-3 proteins were also known to be involved in mTOR pathway regulation under hypoxia through associating with mTOR inhibitory proteins (DeYoung et al., 2008).

Notably, mass spectrometry analysis also identified aspirin induced the hypophosphorylation of serine 44 of TIF-IA, a site known to be dephosphorylated by mTOR activity inhibition (Mayer et al., 2004). Consistent with this, I found aspirin-induced TIF-IA depletion was completely abrogated in the presence of phosphatase inhibitor calyculin A, suggesting phosphorylation status alterations were involved in

this regulation. A previous study has shown that aspirin inhibits mTOR activity (Din et al., 2012), which would suggest an aspirin-mTOR-TIF-IA/S44 dephosphorylation regulation cascade regulates the degradation of this protein. However, I applied the mTOR inhibitor--rapamycin, it caused an increase in the basal TIF-IA protein levels and had no effect on aspirin-mediated TIF-IA degradation (Figure 4.8 B). This implies aspirin may simultaneously regulate other kinase pathways in addition to mTOR for the modification of serine 44 on TIF-IA and the regulation of TIF-IA degradation. In addition, further experiments using the cells have constitutively activated mTOR pathway would help to understand the involvement of mTOR inhibiton in this regulation. Furthermore, since mass spectrometry results suggest the serine 44 is the only phosphorylation site modulated by aspirin treatment, it will be very interesting to use the phosphomimetic mutations constructs on this residue (Figure 6.3) to understand whether this mutations blocked aspirin's effects on nucleolar stress, the NF-κB pathway activation and even aspirin-induced cancer cell apoptosis. Mechanistically, it will be also interesting to know whether this residue is important for TIF-IA-p14ARF binding.

In summary, based on this preliminary finding from mass spectrometry analysis, future studies with detailed investigation would help to identify signals that regulate p14ARF-TIF-IA binding and the interaction partners/post-translational modifications that selectively target TIF-IA for proteasomal or lysosomal degradation under aspirin treatment.

Chapter 7: Discussion

Based on the previous observations in the lab that aspirin and other stresses effects on nucleolus precede stimulation of NF-κB pathway, this thesis primarily aims to establish the molecular connection between stress signalling, nucleolar response and NF-κB modulation. With a view to build on the molecular mechanisms underlying the pro-apoptotic effect of aspirin, which has been established in the host laboratory. The work presented in this thesis generally achieved these goals and has great significance, as I demonstrate for the first time that nucleolar stress can activate NF-κB signalling pathway and characterise a novel nucleolar stress response pathway that lies upstream of this activation. Furthermore, I demonstrate that this pathway is involved in aspirin-induced apoptosis, which together with the observations in the lab indicating the clinical significance of this pathway, propose a novel mechanism of anti-tumour action of aspirin.

Summarised work in this thesis, I propose the nucleolar stress response model outlined in Figure 7.1. I suggest that stress stimuli of NF-κB (including aspirin, UV-C radiation and ceramide), directly or indirectly through inhibiting CDK4 kinase activity, enable TIF-IA-p14ARF interaction within the nucleolus. The de-phosphorylation on serine 44 of TIF-IA could be an important modification for this interaction. I also suggest p14ARF then transports TIF-IA outside nucleolus for degradation. UBF acts as an accessory and phosphorylation status on serine 484 of UBF is essential for this process. As a consequence of this nucleolar disruption in response to stress, specific proteins are probably released from nucleoli into the cytoplasm, where they induce degradation of IκBα and activation of NF-κB driven transcription.

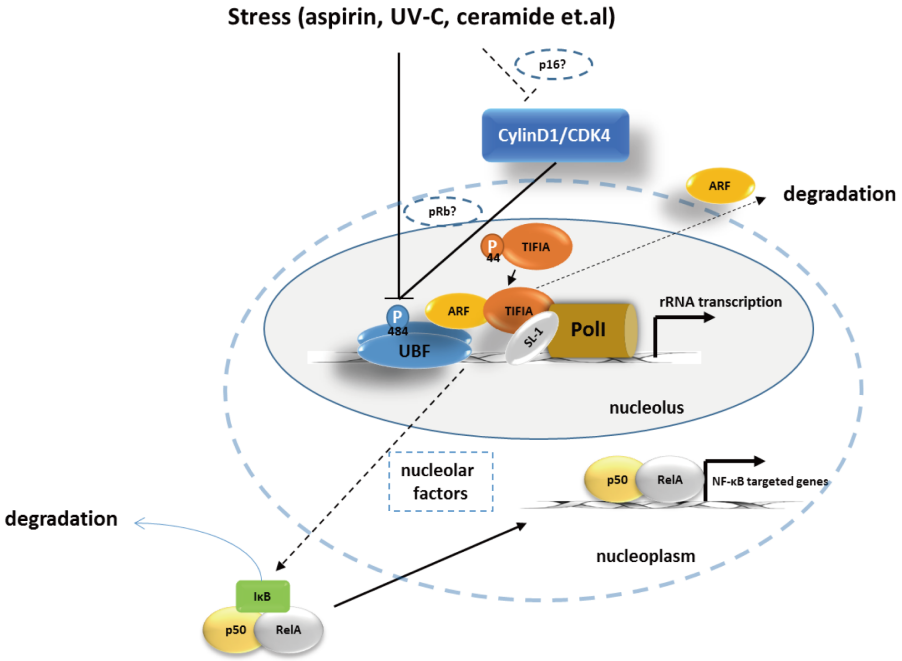


Figure 7.1 Schematic model shows a novel nucleolar stress response pathway that culminates in activation of NF-κB.

Based on my data above, I proposed that stress, directly or indirectly through inhibition of CDK4, triggers p14ARF facilitated degradation of TIF-IA. UBF acts as an accessary in this process. I propose that degradation of TIF-IA cause a specific type of nucleolar stress (characterised by enlarged nucleoli), which results in activation of the cytoplasmic NF- κB pathway and ultimately modulation of the NF- κB controlled gene transcription programme.

Pol I complex disruption activates the NF-κB pathway

Compared to conventional activation of NF-κB induced by cytokines such as TNFα, stresses stimulate the NF-κB with a delayed kinetics. Therefore, stress stimulation of NF-κB could go through a different pathway involving components that are distinct from the known factors/kinases participating in the cytokine-activated NF-κB. Preliminary work in the lab had evidenced that stress-induced disruption of nucleoli preceded the activation of NF-κB pathway and nucleolar translocation of NF-κB RelA (Figure 1.10). They also interestingly found almost all the stress stimuli of NF-κB modulate nucleolar functions based on a literature review (Table 3.1). These findings strongly suggest a casual link between these two events.

In chapter 3, I made the novel observations that nucleolar stress activated the NF-κB pathway. To establish the link between nucleolar disruption and p53, Rubbi and

Miller artificially disrupted the Pol I complex then examined p53 stability (Rubbi and Milner, 2003). Therefore, I firstly used this approach to examine the link between nucleolar disruption and activation of NF-κB. I demonstrated that disrupting Pol I complex, using siRNA to Pol I components induced increased NF-κB activity. This was not observed in cells expressing super-repressor IκBα, indicating Pol I complex disruption stimulates the cytoplasmic pathway (Figure 3.1 and 3.2).

I also observed the Pol I complex disruption regulated the expressions of NF-κB directly targeted genes, and probably with a dynamic property (Figure 3.3-3.5), which was termed as 'NF-κB oscillation' due to the upregulation of IκBα protein level upon NF-κB activation (Nelson et al., 2004). Despite the phenotypic consequences of nucleolar stress activation of NF-κB are not clear based on my gene expression studies, according to my results and the function of NF-κB pathway, I postulate nucleolar stress activation of NF-κB modulating inflammatory response and cell growth/death. Further studies using gene microarray upon inducibly knocking down Pol I componetns would accurately answer this question in depth.

I have yet to identify the molecular mechanism underlying nucleolar disruption activated NF-κB pathway, but I demonstrate this activation is specifically through disruption of Pol I complex rather than a general effect upon inhibition of rDNA transcription. Since the chemical inhibitors targeting rDNA transcription had no effects on the NF-κB pathway (Figure 3.6). Therefore, this would distinguish the mechanism underlying the novel nucleolar stress activation of NF-κB from the well-established nucleolar stress stabilisation of p53. In parallel with the relocation of ribosome proteins (e.g. RPL5/RPL11) results in the stabilisation of p53 as a downstream consequence of nucleolar stress (Deisenroth and Zhang, 2010), I postulate Pol I complex disruption causes the loss of nucleolar integrity and relocation of specific components of Pol I complex or other nucleolar proteins to the cytoplasm where it activates NF-κB signalling pathway. I tested the potential role of CK2 in this regulation, as CK2 is one of the components in the Pol I transcription complex, known to shuttle between subcellular compartments and modulates NF-κB under stress (Bierhoff et al., 2008; Drygin et al., 2010; Kato et al., 2003). I found the activated NF-κB-driven transcriptional activity by siRNA silencing UBF was slightly

repressed in the presence of siRNA against CK2 subunits (Figure 3.6). However, due to the uncertain of CK2 knock-down efficiencies, this data is not conclusive to prove the involvement of CK2 in connecting Pol I complex disruption and NF-κB activation.

Taken together, these data suggest a novel consequence of nucleolar stress is NF-κB activation, allow further studies to pinpoint the phenotypic consequences of nucleolar disruption stimulation of NF-κB and to explore the exact molecular mechanisms by which loss of nucleolar integrity activates NF-κB pathway.

Stress induce degradation of Pol I components, TIF-IA

In order to understand the role of nucleolus in stress-stimulation of NF-κB, I further investigated the molecular changes in the nucleolus in response to stress, and made the novel observation that stress induce degradation of Pol I transcription initiation factor, TIF-IA.

TIF-IA, Rrn3 in mice, is a basal component of the Pol I initiation complex that is indispensiable for regulating cell growth (Jin and Zhou, 2016). In chapter 4, I evidenced certain stress stimuli of NF-κB, such as aspirin, UV-C and ceramide isoforms, induce the reduction of TIF-IA protein level (Figure 4.1 and 4.2). This is the first study to show the depletion of this protein under stress. It is distinct from the reported mechanisms of stress regulations on TIF-IA that inactivate TIF-IA through modulation its phosphorylation status without significant change on its protein level (Drygin et al., 2010).

Although several mechanisms have been considered could explain stress-induced TIF-IA depletion, I demonstrated this depletion is due to stress-mediated protein degradation, rather than inhibition on *TIF-IA* gene transcription or translation (Figure 4.3-4.5). However, our results revealed the known TIF-IA regulation pathways that involve ubiqutination-mediated proteasomal degradation are not applied for this situation. Instead, my study shows for the first time that TIF-IA degraded by lysosome. In addition, only combination both proteasomal and lysosomal inhibitors can completely abrogate aspirin-induced degradation of this protein, suggesting a

cooperation of these two protein degradation pathways under stress condtion (Figure 4.5). Interestingly, there are studies have evidenced the redundancy of the degradation pathway, and shown some proteins are degraded by boths pathways (Lilienbaum, 2013). Futhermore, the fact that only blocking both degradation pathways completely abrogates aspirin effects on TIF-IA would also suggest a hypothesis that proteasome and lysosome targets different kinase or phosphatase upstream of TIF-IA degradation in the presence of this agent. And these two pathways may be required sequentially in the pathway rather than directly targeting TIF-IA simultaneously.

I characterised 94-204 amino acids domain on TIF-IA is sufficient for the stress response of this protein, but the modification sites have yet to be identified (Figure 4.6-4.8). However, finding from the mass spectrometry analysis revealed the phosphorylation on serine 44 of TIF-IA protein was down-regulated by aspirin treatment (Figure 6.2). Serine 44 and serine 199 of TIF-IA are both regulated by mTOR pathway and phosphorylation on these two sites has opposite effects on rDNA transcription and cell function (Mayer et al., 2004). This suggests a hypothesis that aspirin could induce kinase cascades that regulates multiple phosphorylation sites on TIF-IA preceding the degradation of this protein.

I observed the cellular location of TIF-IA was changed under stress, which became disperse in the nucleolus (Figure 4.1). Although some of my data have suggested aspirin induces TIF-IA translocated into cytoplasm where it would be degraded, we have yet to prove it directly from our immunocytochemistry experiments that to see cytoplasmic accumulation of this protein in the presence of aspirin. However, further 3D confocoal with quantifications would help address this question. As reported by a study investigating subcellular localisation of TIF-IA, although nucleolus appeared as the brightest cellular compartment with TIF-IA from the 2D confocal slices, it just occupied 7% of total celluar TIF-IA after quantified under 3D images and taking compartment volume into consideration (Szymanski et al., 2009). Besides, despite the preliminary results in the lab have evidenced stress stimuli of NF-κB exert distinct nucleolar morphological change (increased nucleli size, decreased nucleoli number and nucleolar components segregation), we have yet to characterize whether

these changes drive TIF-IA degradation or *vice versa*. The nature of nucleoli formation is nucleoli forming around rDNA termed nucleolar organizer regions (NORs) and active rDNA transcription are thought to maintain nucleoar structure. Notbaly, there was a study suggested the density of the proteins within nucleoli make it act like a 'liquid droplet', which could simply alter sizes and shapes under stresses by accumulating or dispersing nucleolar factors (Brangwynne et al., 2011). Therefore, one hypothesis in this regard is aspirin could target nucleolar factors that affect the structure of the acrocentric chromosomes, which caused the 'droplet' spread. Further experiments to test the structure of acrocentric chromosomes and the association between TIF-IA and other Pol I factors with rDNA under aspirin stress condition would be with great interest.

UBF-p14ARF-TIF-IA axis links stress signalling, nucleolar response and stimulation of NF-κB pathway

Having found TIF-IA degradation is a response to stress-stimuli of NF-κB, I further demonstrate this degradation is directly linked with stress effects on nucleolus and NF-κB stimulation. Based on the analysis from the previous aspirin-nucleolar SILAC data in the lab, I revealed p14ARF was a regulator of stress-mediated degradation of TIF-IA. Blocking aspirin-induced TIF-IA degradation in the presence of p14ARF siRNA also abrogated the nucleolar enlargement, NF-κB activation and induction of cell apoptosis in response to this agent (Figure 5.2-5.5 and Figure 5.10). Besides, I demonstrated UBF acts as an accessory of this regulation, with phosphorylation status of serine 484 on this protein is required (Figure 5.8 and 5.9). I have yet to illustrate the relationship between UBF and p14ARF in this process, but given the known regulation that p14ARF associates with UBF in nucleolus and modulate its phosphorylation (Ayrault et al., 2006), I propose the phosphorylation status of UBF is essential for the interaction between p14ARF and TIF-IA or the degradation of TIF-IA.

In terms of the upstream pathways regulating stress-mediated nucleolar response, I addressed the involvement of CDK4 kinase in this regulation by showing CDK4 inhibition mimics TIF-IA degradation and shared the similar mechanism of this degradation as aspirin, particularly through modulation on the UBF-p14ARF axis

(Figure 4.9-4.10 and 5.6-5.7). These data are in consistent with the fundamental works in the lab showing CDK4 inhibition replicates aspirin-caused nucleolar enlargement and rDNA transcription inhibition, thus suggesting the importance of CDK4 inhibition in this nucleolar response pathway. Notbaly, I also observed although aspirin-induced TIF-IA degradation can only be abrogated by applying both proteasomal and lysosomal inhibitors, either of it can efficiently block CDK4 inhibitor-induced TIF-IA degradation. This could suggest aspirin triggers a number of pathways, which includes CDK4-mediated pathway. However, whether CDK4 is a direct regulator of aspirin-induced stress response need to be further addressed.

From my results, I can not completely rule out the possibility that other factors/pathways besides UBF/p14ARF are involved in this regulation. Firstly, depletion of UBF/p14ARF did not completely block aspirin/CDK4i-mediated TIF-IA degradation. Secondly, aspirin/CDK4i causes TIF-IA degradation to a reasonable extent in cells without or low expression of p14ARF. Thirdly, mass spectrometry analysis implies involvement of other signals in this process. However, based on my observations of siRNA against p14ARF significantly blocking aspirin effects on nucleolar morphology, NF-κB activation and cell apoptosis, I propose this UBF-p14ARF-TIF-IA axis is central in regulating stress-stimulation of NF-κB and plays a role in the pro-apoptotic effects of aspirin. I also believe this nucleolar response pathway is not dependent on p53 pathway, which has been well-studied as a regulator in nucleolar stress response. As evidenced in my data that aspirin induced TIF-IA degradation both in p53 wile type or p53 null cells (Figure 4.8 F), and the block effects of siRNA p14ARF on aspirin-mediated TIF-IA degradation do not change in the presence siRNA against p53 (Figure 5.5 F). Furthermore, previous publication in the lab have clarified aspirin effects on NF-κB activation and induction of colorectal cancer apoptosis is unrelated to the p53 status (Din et al., 2004).

The significance of this nucleolar stress response pathway in the pro-apoptotic effect of aspirin.

Nucleolus and Pol I transcription have been emerging as exciting targets for cancer therapy (Reviewed in 1.3.2). Above data I presented, which revealed a nucleolar

response pathway results in the inhibition of rDNA transcription, proposed a novel pathway that could be exploited for therapeutic purpose.

Firstly, our study has found ceramide isoforms mimic aspirin effects on nucleolar morphological change, NF-κB activation and importantly, TIF-IA degradation (Figure 4.2). Interestingly, previous study investigating the anti-tumour effects of aspirin had suggested the inhibition of COX, a direct target of aspirin, mediated cancer cell apoptosis by causing an increase in ceramide levels (Chan et al., 1998). This implies a possibility that the pro-apoptotic effect of aspirin is through upregulating ceramide and subsequently inducing downstream TIF-IA degradation and NF-κB activation.

Secondly, I also suggest that this nucleolar pathway is indeed utilised by aspirin for anti-tumour effects by demonstrating siRNA against p14ARF, which blocks aspirin-mediated degradation of TIF-IA and rDNA transcription inhibition, also blocks the decrease of pro-caspase 3 and induction of colorectal cancer cell apoptosis in response to this agent (Figure 5.10). In supporting with this finding, aspirin treatment in biopsies of fresh resected colon tumour from clinical patients has indicated a strong relationship between aspirin-induced TIF-IA degradation and aspirin effects on phosphorylation of RelA (not published data in the lab). Therefore, this thesis proposes a new mode of the anti-tumour action of aspirin that through modulation on nucleolar response and rDNA transcription inhibition. Further *ex vivo* works in this context would help on the potential utilisation of this model for predicting the cancer prevention outcome of aspirin in individuals. Interestingly, an aspirin-induced genome-wide expression study in colon cancer HT-29 cells strongly support the significance of nucleolar response in anti-tumour action of this agent, as they revealed the most marked effects of aspirin were on genes encoding proteins function on ribosome assembly and rRNA metabolism (Yin et al., 2006).

Furthermore, in my study, I demonstrated CDK4 inhibition mimics aspirin effects on this nucleolar response pathway, that is cause UBF/p14ARF-facilitated TIF-IA degradation, inhibition of rDNA transcription and stimulation of NF-κB pathway. Defects in the cyclin D1/CDK4 pathways is common in human cancer, which result in the uncontrolled G1/S cell cycle progression and lead to excessive proliferation in

tumour cells (Peurala et al., 2013). There is a great interest in targeting CDK4 for cancer therapy. However, most of these inhibitors shown efficiency in inhibition CDK4 activity have unclear anti-tumour mechanism (Sherr et al., 2016). Here, the data I presented suggest CDK4 inhibitor could repress the dysregulated nucleolar activity, thus proposing a novel mechanism by which CDK4 inhibition could reduce cancer cell growth.

Taken together, these works have made steps forward in understanding the molecular basis underlying the anti-tumour effects of aspirin. Based on these new aspects and previously identified molecular mechanisms, I propose a new model that summarise the significance of the host laboratory findings in understanding the anti-tumour action of aspirin, with a view for developing novel therapeutic targets against colon cancer (Figure 7.2). This model also addressed the central role of nucleolus and NF-κB pathway in aspirin response.

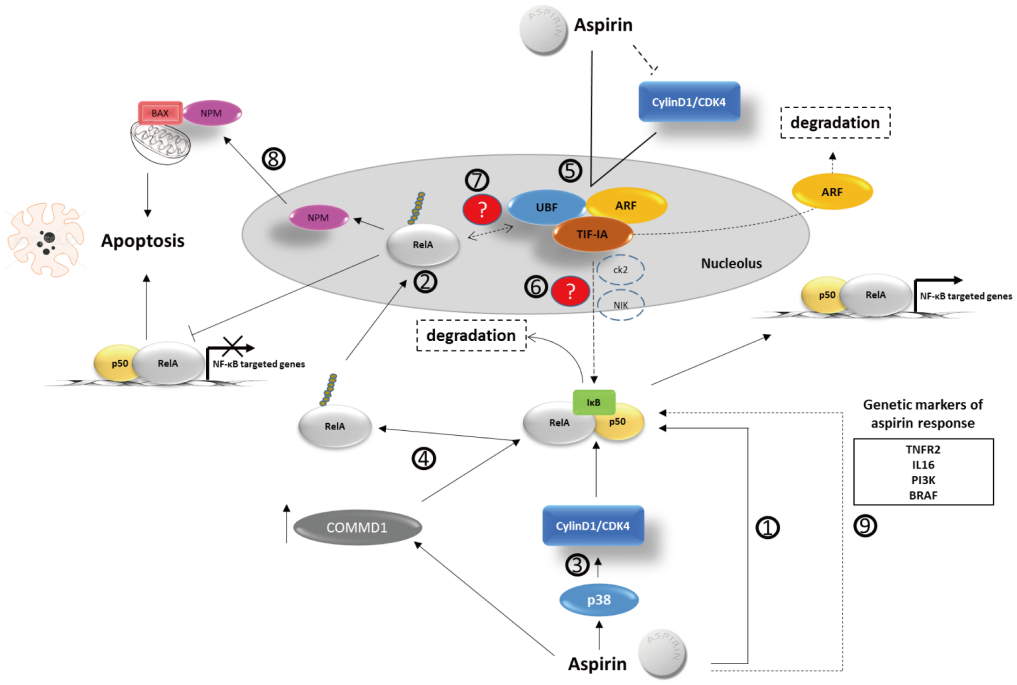


Figure 7.2 The central role of nucleolus and NF-κB signalling in anti-tumour activity of aspirin

1) Aspirin induces degradation of IκBα, NF-κB nuclear translocation, which is linked with cell growth inhibition (Stark et al., 2001).

2) NF-κB component RelA is sequestered in the nucleolus following aspirin treatment, as well as other stimuli such as UV-C and serum starvation, but not by cytokines like TNFα and TRAIL. As a result of this sequestration, NF-κB driven transcriptional activity in nuclear is repressed and NF-κB targeted genes expression is decreased, which consequently induce cell apoptosis. A N-terminal motif (27-30 amino acids) of RelA has been identified as nucleolar localisation signal (NoLS) (Stark and Dunlop, 2005).

3) Upstream molecular pathway responsible for nucleolar targeting of RelA under aspirin stress was characterised (Thoms et al., 2007b). The host laboratory revealed aspirin stimulated the p38 mitogen-activated protein kinase (MAPK) pathway in prior to degradation of Cyclin D1 and inhibition of CDK4 activity. They also demonstrated this upstream signalling cascade preceded the activation of NF-κB pathway and nuclear/nucleolar translocation of RelA. CDK4 inhibition reproduced aspirin's effects on NF-κB pathway and cell apoptosis through a common mechanism involving the N-terminal NoLS of RelA (Thoms et al., 2007b).

4) Molecular mechanisms underlying aspirin-induced RelA nucleolar accumulation were further determined. That is, ubiquitination of RelA is essential for this process and NoLS on this protein is critical for ubiquitination modification. Moreover, a scaffold protein COMMD1was identified interacts with RelA and promotes its ubiquitination following aspirin treatment (Thoms et al., 2010). Further study also revealed aspirin upregulates COMMD1 protein level through promoting p300-mediated acetylation of COMMD1 which protects COMMD1 from XIAP-mediated proteasomal degradation (O'Hara et al., 2014).

5) In this thesis, I demonstrated aspirin/CDK4 inhibition induced degradation of Pol I factor TIF-IA through regulation on a UBF-p14ARF axis. And this nucleolar stress response pathway lies upstream of stress-mediated activation of NF-κB and cell apoptosis.

6) The mechanism of how nucleolar disruption activates NF-κB has yet been characterised, with some potential candidate factors been proposed for further study.

7) Based on previous and my studies, I propose the nucleolus is central for cellular aspirin response. First, aspirin-induced nucleolar stress response precedes its effect on activation of cytoplasmic NF-κB. Second, nucleolar sequestration of RelA NF-κB is a downstream consequence of aspirin treatment and directly linked with cell apoptosis. It would be bold but interesting to hypothesis there was a link between this novel nucleolar stress response pathway identified in this thesis and nucleolar sequestration of RelA in response to aspirin, which formed a feedback loop to regulate the activity of NF-κB pathway and anti-tumour effects of aspirin.

8) Previous study in the lab also indicated another pathway mediated aspirin-induced cell apoptosis which was not dependent on NF-κB driven transcription activity. They found nucleolar accumulation of RelA promoted relocation of nucleolar protein NPM to the cytoplasm, which consequently facilitated the accumulation of BAX in mitochondrial and resulted in enhanced cell apoptotic activity (Khandelwal et al., 2011).

9) The knowledge of genetic markers that could predict the anti-tumour effects of aspirin in individuals will be essential to forward the cancer-prevention application of aspirin into personalized clinical assessment and practice (see Chapter 1 section 1.6). Considering the close relationships between the NF-κB pathway and current identified genetic markers (TNFR2, IL-16, PI3K, BRAF, Chapter 1 section 1.6), it would be interesting to know whether the activation of NF-κB is directly related to the outcome of aspirin treatment in colorectal cancer prevention.

In summary, work carried out in this thesis demonstrated for the first time that nucleolar stress activates NF-κB pathway. I also illustrated how the nucleolus appears to be central in the cells response to stress stimuli of NF-κB and a novel nucleolar pathway containing CDK4-UBF-p14ARF-TIF-IA axis was identified in this regard. My work has already highlighted the complexity of this nucleolar stress response pathway and addressed the orientations for further investigations in elucidating this pathway. Furthermore, I indicated the potential role of this pathway

in the anti-tumour effects of aspirin, and believe future works are worthwhile to be focused on deeply understanding the role and its significance in clinical practice.

Conclusion – nucleolus is central for sensing stress-stimuli of NF-κB which is most likely utilised by aspirin to kill colon cancer

Appendix

Table 8.1. List of proteins interact with TIF-IA in the absence of aspirin.

p value	IP/ control ratio	Peptides	Unique peptides	Unique sequence coverage (%)	Majority protein IDs	Protein names	Gene name
8.20E-07	3.90E+06	1	1	1.6	Q15061	WD repeat-containing protein 43	WDR43
2.51E-05	1.94E+02	4	4	12.7	Q3B726	DNA-directed RNA polymerase I subunit RPA43	TWISTNB
5.07E-05	4.29E+01	9	9	49.5	P19388;A0A087WVZ9;A0A0A0MQR7;A0A087WWX0	DNA-directed RNA polymerases I, II, and III subunit RPABC1	POLR2E
8.66E-05	1.63E+03	39	39	25.5	O95602;B9ZVN9	DNA-directed RNA polymerase I subunit RPA1;DNA-directed RNA polymerase	POLR1A
1.09E-04	3.17E+07	2	2	17	U3KPY1;U3KQB0;P61218;U3KQS8;F8WC47;B0QYL8;B0QYL9	DNA-directed RNA polymerases I, II, and III subunit RPABC2	POLR2F
1.32E-04	2.57E+07	6	6	12	O15446	DNA-directed RNA polymera	CD3EAP

						se I subunit RPA34	
1.50E-04	**1.90E+02**	**21**	**21**	**26.1**	**F5H148;Q9NYV6;H3BMT1**	**RNA polymerase I-specific transcription initiation factor RRN3**	**RRN3**
1.94E-04	2.39E+01	5	5	16	CON__ENSEMBL:ENSBTAP00000024466;CON__ENSEMBL:ENSBTAP00000024462		
1.98E-04	1.67E+08	16	16	12.5	Q9H9Y6	DNA-directed RNA polymerase I subunit RPA2	POLR1B
2.54E-04	2.45E+07	1	1	10.7	C9JLU1;P52434	DNA-directed RNA polymerases I, II, and III subunit RPABC3	POLR2H
2.68E-04	7.45E+07	2	2	13.8	P53803	DNA-directed RNA polymerases I, II, and III subunit RPABC4	POLR2K
2.76E-04	3.53E+07	5	5	7.1	Q9GZS1	DNA-directed RNA polymerase I subunit RPA49	POLR1E
2.79E-04	1.67E+07	5	3	19.1	E7EX70		POLR1E
2.85E-04	3.84E+07	2	2	30.3	Q7Z776;A0A087X0U2;Q9Y2S0	DNA-directed RNA	POLR1D

						polymerases I and III subunit RPAC2	
5.59E-04	7.61E+02	15	15	42.5	O15160;E7EQB9	DNA-directed RNA polymerases I and III subunit RPAC1	POLR1C
1.09E-03	2.61E+01	2	2	31.9	CON__ENSEMBL:ENSBTAP00000014147		
1.54E-03	3.65E+00	3	3	2.8	A0A087WTA8;P08123	Collagen alpha-2(I) chain	COL1A2
2.30E-03	8.14E+06	3	3	29.9	P62875	DNA-directed RNA polymerases I, II, and III subunit RPABC5	POLR2L
2.45E-03	2.17E+06	2	2	4.4	D6REL5;Q6UWZ7	BRCA1-A complex subunit Abraxas	FAM175A
5.11E-03	9.57E+00	1	1	24	J3QL07;J3QLL0;J3KS65;P52292	Importin subunit alpha-1	KPNA2
5.23E-03	3.02E+00	4	4	3.6	P08238	Heat shock protein HSP 90-beta	HSP90AB1
6.17E-03	2.67E+06	1	1	23.1	F8WEB6;A2IDB1;Q04917	14-3-3 protein eta	YWHAH
2.28E-02	1.03E+01	2	2	8	CON__ENSEMBL:ENSBTAP00000011227;CON__ENSEMBL:ENSBTAP00000033053;CON__Q1RMK2		
2.45E-02	1.11E+06	1	1	5.8	H0YJ11;H0	Alpha-	ACTN1;

					YJW3;H7C5W8;H7C144;F5GXS2;H9KV75;P12814;O43707	actinin-1;Alpha-actinin-4	ACTN4
2.68E-02	2.35E+00	4	4	4.3	P02452	Collagen alpha-1(I) chain	COL1A1
2.87E-02	2.19E+00	1	1	9.4	R4GN98;P06703	Protein S100;Protein S100-A6	S100A6

Table 8.2 List of proteins interact with TIF-IA in the presence of aspirin.

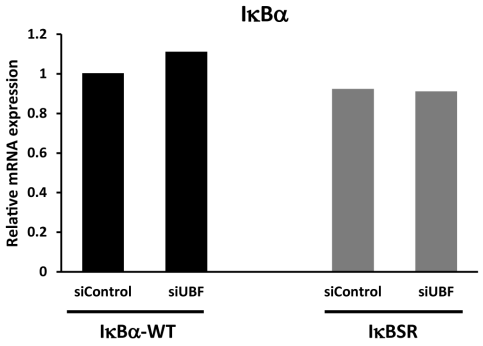
p value	IP/ control ratio	Peptides	Unique peptides	Unique sequence coverage (%)	Majority protein IDs	Protein names	Gene name
1.73E-04	2.62E+06	2	2	4.4	D6REL5;Q6UWZ7	BRCA1-A complex subunit Abraxas	FAM175A
2.10E-04	2.33E+07	2	2	17	U3KPY1;U3KQB0;P61218;U3KQS8;F8WC47;B0QYL8;B0QYL9	DNA-directed RNA polymerases I, II, and III subunit RPABC2	POLR2F
3.48E-04	4.20E+00	3	3	2.8	A0A087WTA8;P08123	Collagen alpha-2(I) chain	COL1A2
3.70E-04	2.36E+07	2	2	30.3	Q7Z776;A0A087X0U2;Q9Y2S0	DNA-directed RNA polymerases I and III subunit RPAC2	POLR1D
4.06E-04	1.19E+02	2	2	31.9	CON__ENSEMBL:ENSBTAP00000014147		
1.10E-03	**5.48E+02**	**21**	**21**	**26.1**	**F5H148;Q9NYV6;H3B**	**RNA polymer**	**RRN3**

					MT1	**ase I-specific transcription initiation factor RRN3**	
1.19E-03	4.58E+01	5	5	16	CON__ENSEMBL:ENSBTAP00000024466;CON__ENSEMBL:ENSBTAP00000024462		
1.32E-03	3.15E+08	39	39	25.5	O95602;B9ZVN9	DNA-directed RNA polymerase I subunit RPA1;DNA-directed RNA polymerase	POLR1A
1.83E-03	9.15E+00	1	1	3.3	P47985;P0C7P4	Cytochrome b-c1 complex subunit Rieske, mitochondrial;Cytochrome b-c1 complex subunit 11;Putative cytochrome b-c1 complex subunit Rieske-like protein 1	UQCRFS1;UQCRFS1P1
2.18E-03	2.22E+01	9	9	49.5	P19388;A0A087WVZ9;A0A0A0MQR7;A0A087WWX0	DNA-directed RNA polymerases I, II, and III subunit	POLR2E

						RPABC1	
2.59E-03	3.35E+00	4	4	4.3	P02452	Collagen alpha-1(I) chain	COL1A1
3.01E-03	9.87E+07	16	16	12.5	Q9H9Y6	DNA-directed RNA polymerase I subunit RPA2	POLR1B
3.59E-03	1.60E+07	6	6	12	O15446	DNA-directed RNA polymerase I subunit RPA34	CD3EAP
3.85E-03	1.38E+01	2	2	8	CON__ENSEMBL:ENSBTAP00000011227;CON__ENSEMBL:ENSBTAP00000033053;CON__Q1RMK2		
4.74E-03	1.09E+02	2	2	13.8	P53803	DNA-directed RNA polymerases I, II, and III subunit RPABC4	POLR2K
8.50E-03	3.26E+01	5	5	7.1	Q9GZS1	DNA-directed RNA polymerase I subunit RPA49	POLR1E
1.55E-02	4.18E+07	4	4	12.7	Q3B726	DNA-directed RNA polymerase I subunit RPA43	TWISTNB
2.87E-02	3.83E+02	15	15	42.5	O15160;E7EQB9	DNA-directed RNA polymera	POLR1C

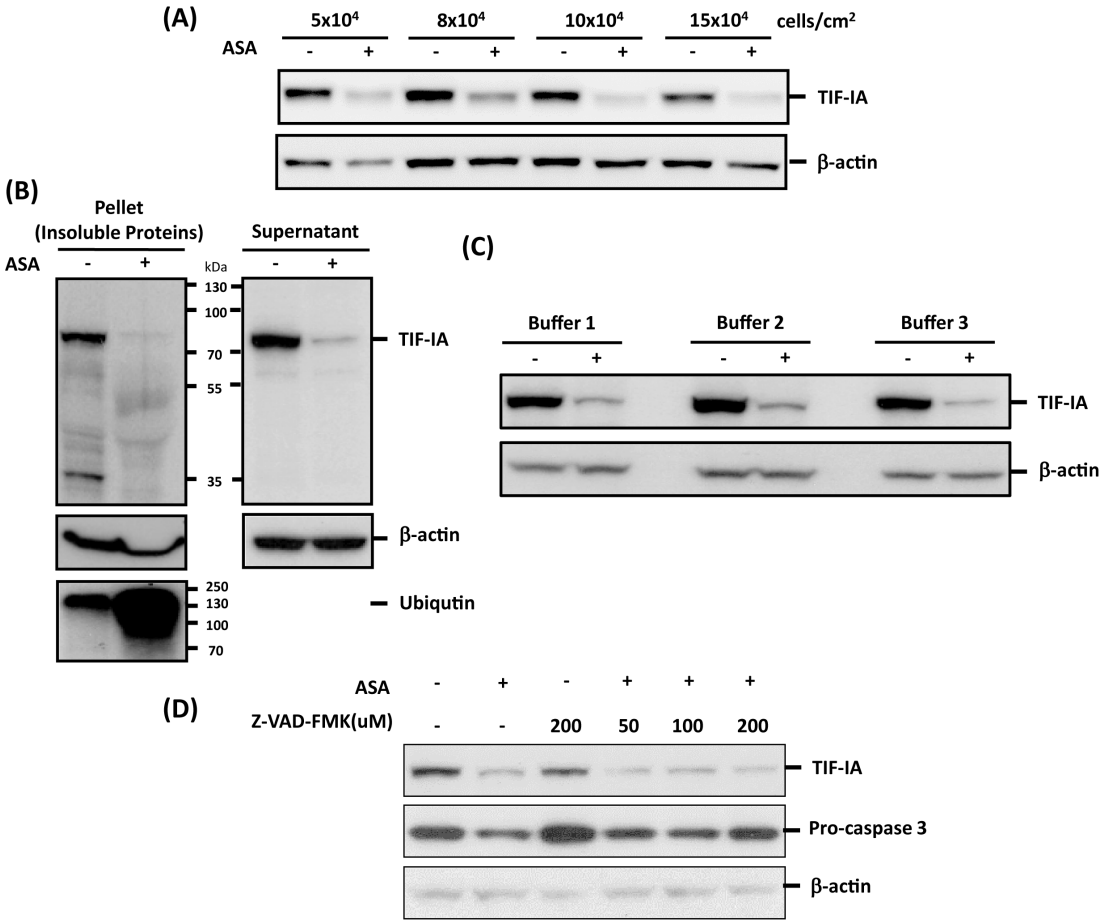
						ses I and III subunit RPAC1	
3.00E-02	5.12E+00	1	1	7.1	A0A0D9SF20;K7EKJ7;P26599	Polypyrimidine tract-binding protein 1	PTBP1
4.16E-02	4.46E+00	1	1	4.4	Q13442	28 kDa heat- and acid-stable phospho protein	PDAP1

Supplementary Figures



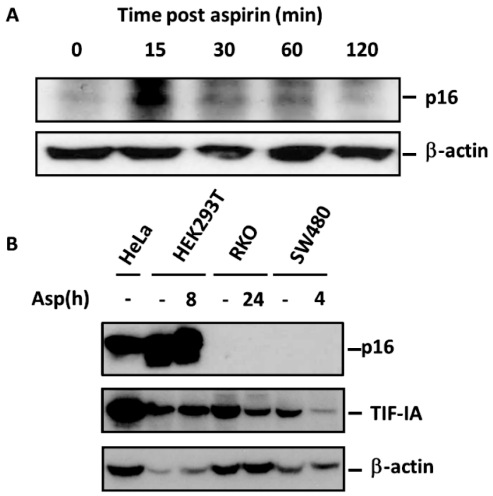
Supplementary Figure 3.1 Knocking down UBF induced *IκBα* gene expression upregulation is dependent on the phosphorylation and degradation of IκBα

SW480 cells were co-transfected with indicated siRNA and 3ug IκBα wild type construct or IκBSR construct. 48 hours after transfection, total RNA were extracted for cDNA synthesis and qRT-PCR taqman gene expression analysis. Relative level of IκBα transcript was calculated comparing to the siControl-IκBα-WT cells (calculated using ddCt algorithm). N=1 independent experiment.



Supplementary Figure 4.1 Aspirin-induced TIF-IA depletion is not affected by cell confluence, not due to alter the solubility of this protein, or through caspase dependent cleavage.

(A) SW480 cells were exposed to indicated concentration of aspirin for 48 hours. TIF-IA protein levels were detected by western blot analysis. **(B)** SW480 cells were non-treated or treated with aspirin (10mM) for 4 hours. Cells were lysed using disruption buffer (20mM Tris/Hcl PH7.5, 2mM EDTA, 150mM NaCl, 1.2% sodium deoxycholate, 1.2% TritonX-100, 1.2% β-mercaptoethanol) then whole lysis was sonicated for 7 seconds x 7 times with 15 seconds break between. Centrifuge at 13000rpm for 15 minutes to harvest pellets and supernatants separately, and then used them for western blot analysis. Antibodies against TIF-IA and ubiquitin were applied. β-actin acts as a loading control. **(C)** SW480 cells were treated with aspirin 10mM for 4 hours. Cells were harvested and lysed using different buffers. Western blot analysis shows the protein level of TIF-IA. **(D)** SW480 cells were pre-treated with indicated concentration of Z-VAD-FMK for 1 hour followed by aspirin (5mM, 16 hours) treatment. Whole cell extracts were prepared, TIF-IA and pro-caspase 3 levels were analysed by western blotting.



Supplementary Figure 4.2 Aspirin could potentially upregulate p16 in colon cancer cells.

(A) SW480 cells were exposed to 10mM aspirin for the indicated time. Western blotting shows the protein levels of p16. **(B)** Indicated cell lines were treated with 5 mM aspirin for the indicated times. Whole cell extracts were prepared, TIF-IA and p16 levels were analysed by western blotting. β-actin acts as a loading control.

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